MGIPC—S1—6 AR/54—7-7-54—10,000.

MODERN METHODS OF MICROSCOPY

A Series of Papers Reprinted from 'Research'

Edited by
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Wallsend-on-Tyne

LONDON
BUTTERWORTHS SCIENTIFIC PUBLICATIONS
1956

BUTTERWORTHS PUBLICATIONS LTD.
88 KINGSWAY, LONDON, W.C.2

AFRICA: BUTTERWORTH & CO. (AFRICA) LTD.
DURBAN: 33/35 Beach Grove

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NEW ZEALAND: BUTTERWORTH & CO. (AUSTRALIA) LTD.
WELLINGTON: 49/51 Ballance Street
AUCKLAND: 35 High Street

Distributed in the U.S.A. by
INTERSCIENCE PUBLISHERS, INC.
250 Fifth Avenue, New York 1, N.Y.

Printed in Great Britain by R. J. Acford Ltd., Industrial Estate, Chichester

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MODERN MICROSCOPY

Introduction

A. E. J. VICKERS

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A series of articles on modern microscopy will be published in forthcoming issues of RESEARCH. *In this introduction, Dr Vickers provides a brief outline of the various types of instruments and methods now available, which will be elaborated in the succeeding contributions. The articles will deal with most aspects of microscopy including microscopic interferometry, the reflecting microscope and its applications outside the visible spectrum, new developments in electron microscopy and the flying spot microscope.*

ALTHOUGH the microscope was invented in the early part of the seventeenth century, it was not until the middle of the nineteenth century that the instrument began to be used for research. The research of LOUIS PASTEUR into silk worm disease and his many investigations in technical biology and biochemistry which followed his bacteriological discoveries established the microscope as the principal instrument of research in the biological field.

A paper by H. C. SORBY in 1864 on the peculiar microscopic structure of iron and steel laid the foundation of the science of metallography. Today metallurgical firms are the largest industrial users of microscopes. Sorby, who may justly claim to be regarded as the father of metallography, can also be considered the father of petrology as it was due to his work that the science of the study of thin sections of rocks was established.

The microscope is still the most important single instrument for research—it is indeed the symbol of science.

THE LIGHT MICROSCOPE

The ordinary instrument consists of a stand designed to hold a condenser which collects light and brings it to a focus within the object studied, an arrangement for holding or supporting the object studied, an objective which when in focus on the object forms an image that is enlarged by the eyepiece. Condenser, objective and eyepiece are so supported that their optic axes coincide.

The value of the microscope as an instrument of precision is determined by resolution, that is, the smallest distance apart of two points which can be recognized as two points. This resolution depends directly on the wavelength of the light used and inversely on the numerical aperture of the combination of objective and condenser employed in the microscope.

Biology, ceramics, metallurgy, mining, paint, paper, textiles, pharmacy and photography are some of the fields which have developed new uses of the microscope and which now regard the instrument as essential to control and research. It is well to pay tribute to the advances in microscope optics made by British makers. H. POWELL's original stands and apochromatic lenses brought British microscopes to the forefront over a hundred years ago ; as a result of new computations of objectives and new methods of testing objectives (especially the interferometric method) this leading position is maintained today.

The shortest distance between two points capable of being resolved is half a wavelength ($0{\cdot}5\lambda$).

A limitation on this distance is imposed by the optical system used to transmit the light; alteration in the numerical aperture of the optical system affects resolution—the greater it is the better the resolution. The cone of light entering the objective affects resolution because the larger the disk of light produced, the smaller the radius of the diffraction disk. If n is the refractive index of the medium from which light enters the objective and U the half-angle of the cone, then $n \sin U = NA$, NA being the numerical aperture. The radius of the diffraction disk is $0{\cdot}5\ \lambda/n \sin U$ which equals $0{\cdot}5\ \lambda/NA$.

Resolution is, therefore, limited by the expression $\lambda/2NA$, *i.e.* it is greater the shorter the wavelength of light used and the larger the value of NA (or any of the factors U or n or $\sin U$ which go to make up NA).

During and since the 1939–45 war there have been considerable advances in the optical construction of the microscope; the quality and uniformity of objectives, condensers and eyepieces have increased, and many new uses of the microscope in research and control have been developed. Indeed, any of the recent exhibitions presented by the Industrial Section of the Royal Microscopical Society show this: agriculture, metallurgy, medicine, chemistry, pharmacy, mining and brewing have all developed special methods in the use of the microscope to elucidate problems and to control operations.

ULTRAVIOLET LIGHT MICROSCOPY

The first optical advance in microscopy was to take advantage of the increase in resolution obtainable by using light of short wavelengths, resolution depending directly on wavelength. A. KÖHLER in Germany designed the first usable ultraviolet light microscope. He employed selected pieces of crystalline quartz from which to fabricate the objectives, but the apparatus was difficult and somewhat dangerous to manipulate. It was not until fused quartz of a quality comparable with optical glass became available about 1920 that the advances in design made by J. E. BARNARD, a hatter of Bond Street whose hobby was microscopical biology, and his friends and colleagues became possible. They evolved the first practical ultraviolet light microscope. It was made by R. and J. Beck Ltd of London and is still in use at the National Institute of Medical Research. The Barnard microscope had numerous mechanical improvements to deal with the increased resolution: the devices for focusing were ingenious, rendering the instrument safe and precise in use.. Among Barnard's collaborators were Sir HERBERT JACKSON, J. SMILES, A. J. PHILPOT, H. MOORE and R. J. BRACEY. There are now on the market a number of instruments which are developments of the Barnard-Beck instrument, an excellent one being that produced by Cook, Troughton and Simms of York. The Barnard microscope was a refraction instrument. Advances in the design and stability of conventional microscopes have made it possible to use ultraviolet optical equipment on an ordinary stand without much trouble. Such apparatus is in use, with excellent results, by A. F. HUGHES in Cambridge.

More recently attention has been given to reflecting microscopes. These have some advantage over the refractors in being achromatic throughout the spectrum from the infra-red through the visible to the ultraviolet region. Two types of instrument have been produced—that by C. R. BURCH in Bristol which is mechanically very complex but a remarkably efficient instrument nevertheless, and that designed by M. F. H. WILKINS' group of the Medical Research Council unit at King's College, London. The objectives designed by the latter are efficient and, while not using aspherical surfaces as is the case with the Burch objectives, they are much less costly and can be used for the same purposes. Their resolution is high and they can be accommodated on a conventional stand. These objectives are made by R. and J. Beck Ltd.

Some extraordinary and exciting work is available to the user of the new reflection objectives. Observation can be made of a living cell in the visible part of the spectrum, photographs may be taken of the same cell while alive in the ultraviolet, visible and infra-red parts of the spectrum, a spectrophotometer can then be attached to the microscope, and absorption spectra of the contents of the cell can be studied in the ultraviolet, visible and infra-red regions with the cell still alive. Thus knowledge of the chemical changes within the cell can be obtained while it is alive and under observation on the microscope stage. These new methods are yielding valuable information on both normal and pathological growth.

FLUORESCENT MICROSCOPY

It is well known that certain types of material will react with a beam of light in such a way that, in passing through the material, the wavelength of the light is increased. The most useful application of this phenomenon is when a beam of ultraviolet light reacting with a suitable material is returned to the eye as a beam of visible light; the light emerging after such a reaction is usually coloured. All kinds of living materials and many minerals show this phenomenon. It is used in microscopy in several ways.

The sub-stage condenser of the microscope is made of quartz so as to be transparent in the ultraviolet region, and the beam of ultraviolet light is sent through it, and usually through a quartz microscope slip supporting the object, to react with the object producing characteristic fluorescent colours. From this point the object fluorescing in ultraviolet light may be examined by the conventional optical system. In some cases a filter is desirable in the eyepiece in order to prevent damage to the eye by such ultraviolet radiation as passes through the system without being absorbed in the glasses used for the construction of objectives and eyepieces.

Fluorescence microscopy has been known for a long time. Not much use was made of it until interest was revived by the work of M. HAITINGER in Vienna. He showed that the methods of fluorescence microscopy could be used in diagnosis, in the study of living tissue, in the study of absorption in various types of tissue and even in morbid histology in the operating theatre. He investigated many fluorochroming dyes—chemicals which when absorbed by tissue and illuminated in the ultraviolet region give rise to characteristic colours. More recently, renewed consideration of Haitinger's work has re-established the fluorescence method of examination. It has been shown that pathological tissue selectively absorbs some of the fluorochroming chemicals in much the same way as it does radioactive tracers. Fluorochromes can therefore be injected into the blood system and the presence of pathological tissue detected by the concentration of the fluorescing dye. The rapid preliminary diagnosis of some forms of cancer can be effectively carried out in this way. Particularly suitable among the dyes in this field of cancer determination are those of the berberine alkaloids. The use of the method in surgery has been advanced by the discovery of non-poisonous fluorescing dyes, such as fluorescine amine, which can be injected into the spine and which are selectively absorbed in tumorous tissues in the brain. When illuminated by ultraviolet light this tissue will show where the concentration of the dye exists. A further example of the use of fluorescence microscopy is the rapid detection of tuberculosis and leprosy bacteria merely by placing the suspected sample on the microscope stage and illuminating with ultraviolet radiation. Rapid and more positive diagnosis can be effected by the use of fluorescing stains such as auramin, which give very characteristic colours to bacteria.

DARK GROUND ILLUMINATION

In using the microscope not only is resolving power combined with the necessary magnification important, but the visibility of the object under observation has to be considered. Visibility is dependent on the contrast between the object and its background. This is shown to some extent by the necessity of staining objects and by the use of coloured screens to reduce glare in the ordinary light microscope.

L. C. MARTIN and B. K. JOHNSON have quoted as a characteristic example of the inability to see an object due to lack of contrast, the spider's web found in the garden. If this is viewed against the sky it is practically invisible, but seen illuminated by sunlight against a dark background it becomes readily visible. There are many microscopic transparent and semi-transparent objects similar to the spider's web, and the general principle of illuminating such objects against dark backgrounds has for some time been adopted in microscopy.

It should be emphasized that the value of the method is to increase visibility. There is no actual increase in resolving power as regards separation of detail when dark ground illumination is used. A serious drawback to this form of illumination is that it produces confusing diffraction effects round the image and makes the latter difficult to interpret. In order to obtain dark ground illumination with the microscope, direct light which illuminates the object must not enter the objective. The only light entering the microscope must be that which has been scattered or reflected by the object. Thus it is necessary to modify the usual form of apparatus employed for illuminating the object, and a special sub-stage condenser is used. For low power lenses this causes no difficulties—the dark field may be obtained conveniently by interposing a stop in the centre of the ordinary condenser. In this way the direct light is prevented from entering the objective and the object is illuminated against a dark background. For higher power lenses and immersion objectives a special reflecting system has to be used so that the light is sent obliquely through the object supporting slip to reach the object at a very high angle, none of the light entering the objective.

A reflecting form of construction is employed in order to avoid chromatic aberrations which are always present when a refracting system is used for this type of illumination. It is very important that the focus of the dark ground condenser should coincide with the position of the object on the microscope slide and for this reason microscope slides have to be very carefully graded to suit dark ground condensers. The method of dark ground illumination is very useful for rapid diagnostic work in bacteriology and in the study of aquatic life.

PHASE CONTRAST MICROSCOPY

One of the most difficult problems in the use of the microscope is to obtain sufficient contrast when examining material, especially living material, which has a refractive index not far removed from that of the surrounding liquid. F. ZERNIKE, during an investigation of the Abbe diffraction theory of image formation, arrived at a very elegant solution of this problem. When light passes through a transparent medium like water or glass it travels more slowly than it does in air, and therefore needs more time to cover a given distance in glass than it does to cover the same distance in air. The period of the vibrations remaining equal in passing through one medium or another, it follows that the light makes more vibrations on its way through glass than it does when covering the same distance in air. Thus the light will lag in phase. However, if the glass is not absorbing the amplitude will remain equal.

The development of Zernike's ideas into the phase contrast microscope made possible the revelation of details in unstained living cells and other transparent specimens. By utilizing the optical path and absorption differences in the microscope to convert invisible phase changes formed in light passing through such specimens into visible differences in intensity, the contrast in the image can be increased or decreased, or reversed and then increased or decreased. Loss of time and damage to specimen by the usual preparation methods are avoided for no special treatment of the specimen is required, although thin material is to be preferred. When light passes through absorbing material the wave is reduced in amplitude so that the waves are flatter. Such changes are visible and may be recorded photographically.

The phase contrast microscope uses optical path and absorption differences to change the phase into amplitude or intensity differences. The methods of phase contrast microscopy will be dealt with fully in a later paper in this series. It remains here to say that, during the 1939-45 war, advances made by British optical manufacturing companies in the design and manufacture of optical equipment suitable for this method were so rapid that it was available for research before the war had terminated.

Outstanding among the researches carried out has been work on cell division. By taking photographs with the cinematograph camera and using living material, it was possible for the first time to render visible as a continuous living process those various stages in cell division which in the past have had to be pieced together very painfully by the examination of a very large number of stained specimens with the inherent difficulties of interpreting dead, fixed and stained material in terms of living material.

INTERFERENCE MICROSCOPY

The interference microscope provides a method for examining phase changing specimens. The theoretical principle has been known for a considerable time, but its practical value has only been appreciated as the result of experience with the now well established phase contrast method of Zernike. Contrast and interference microscopes both provide intensity contrast images of phase changing specimens by means of optical interference phenomena. The important characteristic property of the interference method is that the mutually interfering beams which produce the contrast are generated by an interferometer system incorporated in the microscope itself: thus dependence on diffraction by the object structure is avoided. Advantages derived from this independence are that contrasts can be obtained from features possessing phase changes which are too gradual to diffract an adequate proportion of the light outside the phase step of a phase contrast microscope, enabling phase gradients as well as sharp changes to be perceived, and that direct illumination need no longer be restricted to a narrow portion of the aperture. The first results in an image which brings out the general properties of the specimen and which bears closer resemblance to that obtained by staining methods, while the second avoids the well known artifacts which result from very severe stopping down, notably the curious images of the sub-stage stop formed by features of the object which are partly out of focus.

Of even greater importance than this are the facilities afforded for measuring the precise amount of phase change produced by any given element in the object. This sensitivity under good conditions may exceed 1/300 wavelength. The interference microscope thus permits both observation and measurement of the various phase changes produced by transparent objects and this alone is extremely valuable. It can give information on such subjects as double refraction of material, the protein content of a living cell or its solid to water ratio. The papers of M. M. SWANN, J. M. MITCHISON, A. F. HUGHES and others of the Cambridge School have shown how useful this quantitative aspect of microscopy can be in biological studies.

POLARIZED LIGHT MICROSCOPY

In the use of polarized light with the microscope, perhaps the first claim must go to the science of mineralogy, for Sorby's original work in this connection dates back to 1864. Since then the

polarizing microscope has developed into the principal instrument of research for the mineralogist and the petrologist. Plane polarized light is radiation in which vibration directions perpendicular to that of propagation are confined to one plane only. The production of such light may be carried out in several ways of which the chief are by reflection from a reflecting surface at the appropriate angle, by transmission through a piece of tourmaline cut perpendicular to the optic axis, by transmission through a nicol prism or modification of this prism and, fourthly, by the use of the recently developed oriented chemical materials such as polaroid. The vibration of the light after leaving such a polarizer is in one plane only. If two of these polarizing devices are arranged in sequence and so oriented that their vibration directions are parallel, light will pass through the system to an eye placed behind the second polarizer, generally known as an analyser, but if one of them be rotated so that this plane vibration is at right angles to the other, no light will pass to the eye. This latter condition is known as cross-nicols, the former being parallel-nicols. It is the appearance of an object placed between the polarizer and analyser which provides the microscopist with a considerable aid in the identification of the specimen.

The progress which has been made in quantitative methods of study led to the development and use of this type of microscope not only in the mineralogical and inorganic chemical field but in all branches of crystal chemistry and biology. It enables the identification of minute particles of crystals to be effected with ease and certainty: fibres and membranes embedded in other tissues may be picked out with rapidity.

The polarizing microscope consists of an ordinary microscope with the necessary polarizing devices fitted. In addition, means of varying the position of the object under examination relative to the beam of polarized light, and of measuring the variation, must be incorporated. Arrangements should be made in the microscope for changes between parallel light and convergent light, for the optical effects are different and specific with different crystal systems. Also arrangements must be available for the insertion of various types of compensating devices and materials so that a whole range of optical properties may be measured on the one instrument. Among those which are most useful and most rapidly determined are extinction angles, refractive indices, birefringence, optic axial angles, vibration directions of light within the material and the type of polarization which the material itself produces on a beam of light.

The use of the polarizing microscope has advanced knowledge of the structure of biological objects to a very great degree; especially has this occurred in the study of subjects such as skeletal muscle, the structure of protein molecules and the direction in which long protein molecules arrange themselves when the muscle is subject to various kinds of reaction. Polarized light optics permits the student to investigate the special microscopic structural characteristics and orientation of various cells and tissues. It may be used qualitatively to detect the presence of oriented structure in a biological system, but it has many quantitative applications which have been developed in recent years and which are leading to an exact knowledge of the chemistry of the living cell.

ELECTRON MICROSCOPY

In 1923 Louis de Broglie announced the dual nature of the electron, namely, that it is a negative particle of electricity which has both mechanical and optical properties. It can be considered both as a particle and as a wave of energy. From these ideas there developed a system of electron optics capable of experimental demonstration. By 1932 these studies had advanced so far that E. Brüche and H. Johannson were able to produce an emission electron microscope, while M. Knoll and E. Ruska had developed the first magnetic electron microscope. In 1938 Siemens Halske of Berlin had an electron microscope on the market. Some of their early instruments are still in existence and give beautiful results: later work has produced many designs with all kinds of refinements. Instruments are made in Great Britain, Holland, Germany, the United States, Canada, France and Switzerland. The principle of the instrument is to have a hot body emitting electrons; this is at a high potential so as to shoot the electrons off in a predetermined direction. The bundle of electrons is brought to a point focus by a circular magnet acting as a lens; the focus is the object under examination. A second magnetic lens acts as an objective, producing an enlarged image of the object which is further enlarged and brought to focus in the plane of a photographic plate or fluorescing screen by means of a third magnet or eyepiece. In this way the image is examined.

The electron microscope has added another two orders to the magnification available to the microscopist. It has proved very useful in the study of small particles, surfaces and metallurgy. It is only now being applied in the more difficult field of biology. Workers such as J. R. G. Bradfield in Cambridge indicate the future usefulness of the apparatus in biological investigations.

RECENT DEVELOPMENTS IN ELECTRON MICROSCOPY

V. E. COSSLETT

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Electron microscopy has made spectacular progress in a comparatively short time. Dr Cosslett reviews the most recent advances in lens design and in the production of commercial instruments. Reference is made to the various techniques for the preparation of the main types of specimen. The electron microscope has already found numerous applications and its potentialities are far from exhausted.

THE first volume of *Research* included an account of the progress of electron microscopy in Great Britain[1]. The subject has expanded rapidly since then—indeed, the first International Conference under the sponsorship of the Joint Commission for Electron Microscopy was recently held in London (16 to 21 July 1954)—so that a new and wider review is timely. The Joint Commission was set up by the International Council of Scientific Unions—with membership from the Unions of Physics, Chemistry, Biology and Crystallography—to provide a measure of coordination in a subject that ramifies through almost all branches of pure and applied science. There are now electron microscope societies in all the leading countries in the world, of which the Japanese and American are the largest, but with Germany and Britain (240 members) not far behind. There are about 1000 electron microscopes in operation throughout the world, excluding the Soviet bloc, and it is known that several Russian models are in production.

The wide interest in this new technique is reflected in the large number of papers (160) offered for the London Conference, in face of the rule that only new and unpublished work should be submitted. Almost half of these were concerned with biological applications and specimen techniques, and 20 per cent with metallurgical and chemical applications and techniques, leaving only 20 per cent on the instrument itself and 10 per cent on fundamental electron optics. In this review attention will primarily be directed to general trends, particularly in lens design, production of commercial instruments and new preparation techniques for the main classes of specimen. This survey should reveal the present state and future potentialities of electron microscopy.

ELECTRON LENS CORRECTION

If the electron microscope were only limited by the same factor as its optical counterpart, namely the wavelength of the illumination, then a resolving power of about 1/50 Å would be expected of it. In fact spherical aberration sets a limit of some few Ångstrom units, and astigmatism caused by imperfections in lens construction adds further confusion. The greatest resolution so far claimed is between 6 and 8Å, but this was only achieved after great pains had been taken to reduce astigmatism to a minimum.

Astigmatism, as a true error of the lens field, is negligible in an electron lens of perfect rotational symmetry, but any departure from this symmetry will introduce image confusion even greater than that arising from spherical aberration. The lens bore must be true to better than 1/100,000 in., if the error is to be less than 10Å, but even perfect machining may be offset by lack of uniformity in the magnetic material of which the pole piece is made. The possibility of correction was first demonstrated by J. HILLIER and E. G. RAMBERG[2] in 1947, using soft iron screws in the pole piece gap. Adjustment of the radial distance of the screws, by trial and error, can restore rotational symmetry to the field. A detailed theoretical study of the nature of these lens defects has been made by P. A. STURROCK[3]. Recently more convenient methods of continuously varying the additional field have been devised, particularly by S. LEISEGANG[4], so that correction can be much more rapidly obtained.

The theory of correction with electrostatic fields was first explored by F. BERTEIN[5] and later experimentally applied by O. RANG[6] and by M. E. HAINE and T. MULVEY[7]. Insulated electrodes—either four, six or eight—are introduced radially into the lens, which may be magnetic or electrostatic, and the potentials applied to them adjusted until they give a field of the right distribution to restore the original lens field to rotational symmetry. The method is much easier to operate than any in which magnetic elements have to be moved mechanically; indeed, with four electrodes a rotation about the lens axis is needed, but is not difficult to effect. The importance of ease and rapidity of correction must be stressed, since the degree of astigmatism in a lens will change continuously in use, owing to accumulation of insulating

deposits on the lens aperture, hysteresis in the lens and possibly effects due to the specimen itself.

The detection and correction of small degrees of residual astigmatism involve careful inspection of Fresnel diffraction fringes around specially selected objects[2]. M. E. HAINE and T. MULVEY[8] have developed a method of using the fringes to test also the occurrence of confusion arising from mechanical vibration or electrical and magnetic instabilities. From this work it appears that these effects set the limit to microscope performance, once astigmatism has been sufficiently corrected. Careful attention is now being paid to the design of the specimen stage, to reduce mechanical vibration so that the object does not move with respect to the photographic plate by more than a few Ångstroms during exposure. Sources of trouble arising from stray electric and magnetic fields are also being checked.

If these hindrances can be removed, the inherent spherical aberration of the lens would limit the resolving power to the order of 4 to 5Å. Detailed investigations by G. LIEBMANN[9] of the properties of existing types of magnetic lens have shown that this is the limit to be expected on the grounds of spherical aberration and diffraction effects alone. As already mentioned, practical performance is now within a factor of 2 of this limit and the next stage—the correction of spherical aberration—is being actively explored.

The fundamental advance in this direction was made by O. SCHERZER[10] in 1947, when he showed that correction could in principle be obtained by introducing lens elements of rotational asymmetry (*Figure 1*—A_1, B_1). These give astigmatism of a known degree, producing two line foci which are mutually perpendicular and lie at different points on the axis. A set of appropriately charged and orientated electrodes of fourfold symmetry, placed at these foci (Z_a, Z_b) would correct spherical aberration in each of the astigmatic planes separately. Scherzer showed that a third correcting set (Z_c) was required to ensure correction in all longitudinal planes; further asymmetric elements (A_2, B_2) are also needed to remove the astigmatism initially introduced. Attempts have been made by Scherzer and his school[11,12] to translate these ideas into practice, and it has at least been shown that the method is fundamentally correct. But it is clear that the numerous lens elements involved have to be accurately aligned, and the voltage on each has to be carefully adjusted. It appears to be difficult to maintain correction once it has been obtained.

On this account, attention has recently been paid to devising a system of correction that would be easier to operate even if more limited in application. The Scherzer system would in principle allow the correction of any degree of spherical aberration in a lens operating at any magnification and voltage. It would be adequate for most purposes to have a lens corrected for a single set of working conditions. J. C. BURFOOT[13] has recently shown that a lens of four electrodes will satisfy these conditions, but that the shape of the electrodes would have to be machined to an accuracy beyond anything yet attainable; alternatively, a simpler electrode form could be adopted, and small trimming voltages applied by a method of trial and error. A solution intermediate between the four-element system and that of Scherzer is in process of investigation by G. D. ARCHARD[14], and it is possible that a more practical experimental arrangement will result.

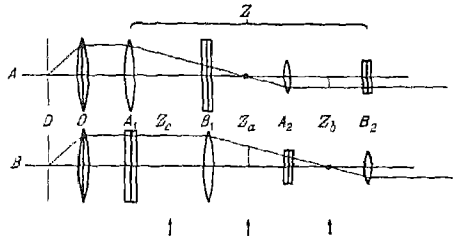

Figure 1. Electron lens system for correcting spherical aberration in vertical (A) *and horizontal* (B) *cross-section: O objective lens; A_1, A_2, B_1, B_2 cylindrical lenses; Z_a, Z_b, Z_c correction electrode assemblies* (*after Scherzer*)

Thus it can be said that the correction of spherical aberration in electron lenses appears much nearer to solution than seemed likely five or six years ago. Attention is also being paid to the elimination of chromatic aberration by the use of similar methods, as Scherzer has shown is possible in principle. In some respects the latter factor may prove more important to practical microscopy than spherical correction, for the extreme experimental requirements of mechanical and electromagnetic stability may make a resolution of a few Ångstroms impossible even with a corrected lens. On the other hand, an achromatic lens would be invaluable for reflection electron microscopy (see below) and for transmission microscopy with thick specimens, since in both cases energy losses in the specimen give the electron beam a chromatic spread, no matter how high its initial degree of monochromatism. Furthermore, the much smaller stabilization required with an achromatic lens would allow a considerable reduction in the cost of the electrical supplies and thus lead to cheaper and more robust commercial models of electron microscope.

INSTRUMENT DESIGN

In recent times there have been two main developments in production models: the introduction, on the one hand, of instruments of limited performance and cost for routine purposes and, on the other hand, of an electron microscope of the highest possible resolving power. The third modern trend towards greater flexibility in operation was already described in the 1948 review[1] apropos of the Metropolitan-Vickers EM3 model. The wide range of magnification and easy transition to electron diffraction made possible by the use of an intermediate stage of magnification, *i.e.* a three-lens instead of a two-lens imaging system, has since been recognized by most other manufacturers of electron microscopes.

An attempt to produce a microscope of limited performance at about half the cost of a standard model was made by the Radio Corporation of America in 1943 in the form of the EMC Console model[15]. But this instrument, whilst having many advantages, suffered from a lack of image brightness and poor resolution. More recently, the Radio Corporation of America has introduced a still smaller model (EMT) in which permanent magnet lenses are used[16]. The microscope column is reduced to no more than twice the length of an optical microscope, and stands on a table with the diffusion pump below it; the electrical controls, backing pump and electrical supplies are contained in separate cabinets. The new model appears to be simple to operate and of adequate resolving power for most routine purposes. Its main defects are the need to replace pole pieces in order to change the magnification (values of 1500×, 3000× and 6000× are available), and some difficulty in alignment. As the strength of the permanent magnet lenses is fixed, focusing is effected by varying the high tension.

The Metropolitan-Vickers junior model (EM4) is more orthodox in design, apart from the fact that the column is horizontal and that a double projector is employed in which a single winding energizes two gaps in the same magnetic circuit. One gap is arranged to saturate at very low excitation, so that variation of the coil current only changes the field strength in the other gap which forms the intermediate lens of a three-lens imaging system. High tension is supplied by a 50kv set employing metal rectifiers, whose dimensions are so small that it is contained in the microscope cabinet which is little larger than a normal office desk and contains even the backing pump. Indeed, it can be held that compactness has here been carried to the point of inconvenience in regard to accessibility of some controls and components. The resolving power is better than 100Å and the flexibility and ease of use are excellent. Either plates or a film camera may be used, and three specimens are simultaneously accommodated in the object holder, so that rapid routine comparison of specimens is possible.

A still more recent model in the same class is the Philips EM75, so named because it works at 75kv, which is approximately the ideal voltage for the average biological or industrial specimen and much higher than that employed in all other routine models. In contrast to the Metropolitan-Vickers transition from a vertical EM3 to a horizontal EM4, Philips have changed from a near-horizontal EM100 to a vertical EM75, and it would appear that the change is for the better in terms of ease of alignment and accessibility of components. The column stands on a desk (*Figure 2*) and terminates in a large cathode ray tube bulb as viewing screen, with the camera between this and the projector lens, as in the EM100. The second projector lens is of novel design, the coil current being maintained constant and the magnetic gap varied by means of an external control, in order to change the lens strength and so

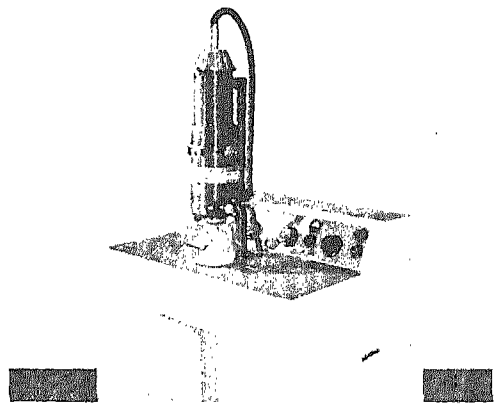

Figure 2. Philips EM75 electron microscope

the magnification. The objective lens is of the new small bore (0·5 mm) and small gap (2 mm) type, which was shown by J. B. LE POOLE and A. C. VAN DORSTEN[17] to have minimum chromatic aberration, thus requiring less refined stabilization of the electrical supplies. By these and other simplifications of design, it has been possible to market the EM75 at about half the price of the standard EM100 model.

The second main trend in instrument design has been towards the production of an electron microscope embodying all the latest improvements and therefore capable of the highest possible resolving power. First in the field is the Siemens-Halske concern, which produced the very first commercial

electron microscope in 1938 from the laboratory model of B. VON BORRIES and E. RUSKA, and now announces the Elmiskop I that has been developed by Ruska over the past few years (*Figure 3*). It includes all the refinements necessary for ease of operation and maximum resolution : adjustable apertures, astigmatism correction, highly stabilized electrical supplies, and a total direct magnification

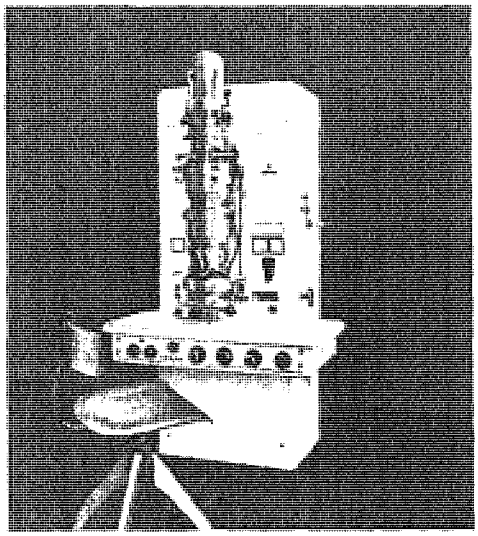

Figure 3. Siemens Elmiskop I electron microscope

of up to 160,000 times to ensure that all the detail inherent in the image is recorded without trouble from photographic grain or electron ' noise '. This very high working magnification is obtained by using a high powered projector and long final image distance giving a third-stage magnification of 250 times, in conjunction with two earlier stages of magnification. The necessary intensity of illumination is ensured by using a double condenser ; the very small electron spot thrown on the object also has the desirable result of minimizing its rise in temperature. Naturally such a machine is considerably more expensive than the average commercial model, but it will render a great service to research if the resolution of the production version approaches the value of 6Å claimed for the prototype. Some of the new developments in specimen technique, mentioned below, can now for the first time provide specimens of scientific interest (as opposed to specially selected test specimens) for which a resolution of 10 to 20Å is demanded. If this range is to be attained regularly, an electron microscope must be capable of considerably better resolution with a test specimen under optimum conditions. Such a performance has been reached by one or two other special laboratory microscopes, in addition to that of Ruska, notably by Haine in the Associated Electrical Industries Research Laboratories[18]. It is to be hoped that the reports are true that his instrument is to be developed into the production stage by Metropolitan-Vickers.

SPECIMEN PREPARATION

Biological techniques—As the literature and recent conference programmes readily show, the biological and medical applications of the electron microscope outnumber all others combined. This is largely due to the fact that ultra-fine details obviously exist in nature between the limit of optical methods and the molecular level, whereas it is not so clear that submicroscopic structure is to be found in metallurgical and industrial specimens—nor whether it is of any relevance to present-day problems, even where it is shown to exist.

Techniques for preparing biological specimens have correspondingly advanced more rapidly than those in other fields. The simplest specimens to handle are those of a particulate nature, such as bacteria and viruses, and many methods of purifying and mounting them in a suitable manner for electron microscopic examination have been given in D. G. DRUMMOND's handbook[19]. Since its publication in 1950, the chief advance has been the application of the spray gun technique by R. C. BACKUS and

Figure 4. Electron micrograph of sprayed droplet of mixture of Rickettsia of pneumonia (rods) and Latex particles (spheres)—magnification × 8000 (courtesy of Dr R. Williams)

R. C. WILLIAMS[20]. The suspension of bacteria or virus is sprayed by air at a moderate excess pressure (20 to 30 lb/in.^{2}) from a miniature gun of glass and metal through a glass jet of bore approximately 0·1 mm. The spray is caught on the normal metal support grids, covered with a nitrocellulose or *Formvar* film. A dispersion of the particles is thus obtained which may be examined by statistical methods, the separate droplets being readily visible in the electron micrograph, especially if the grid has been shadowed with metal. Particle counts can be readily made and quantitative estimation of the concentration in the original suspension deduced (*Figure 4*).

In efforts to shorten and simplify the preparation of suspensions for microscopy, from which all tissue debris or remnants of growth media must be removed if a clear view of the particles is to be obtained, some workers have centrifuged directly on to grids[21,22]. Alternatively, it has been found adequate to pierce the film on the grid with a needle, immediately after depositing the suspension; the bacteria or viruses seem to adhere very rapidly to the film, whilst the debris are carried off with the supernatant liquid[23].

The other main field of biological activity is concerned with bulk tissues, which have to be sectioned for electron microscopy to give even thinner sections

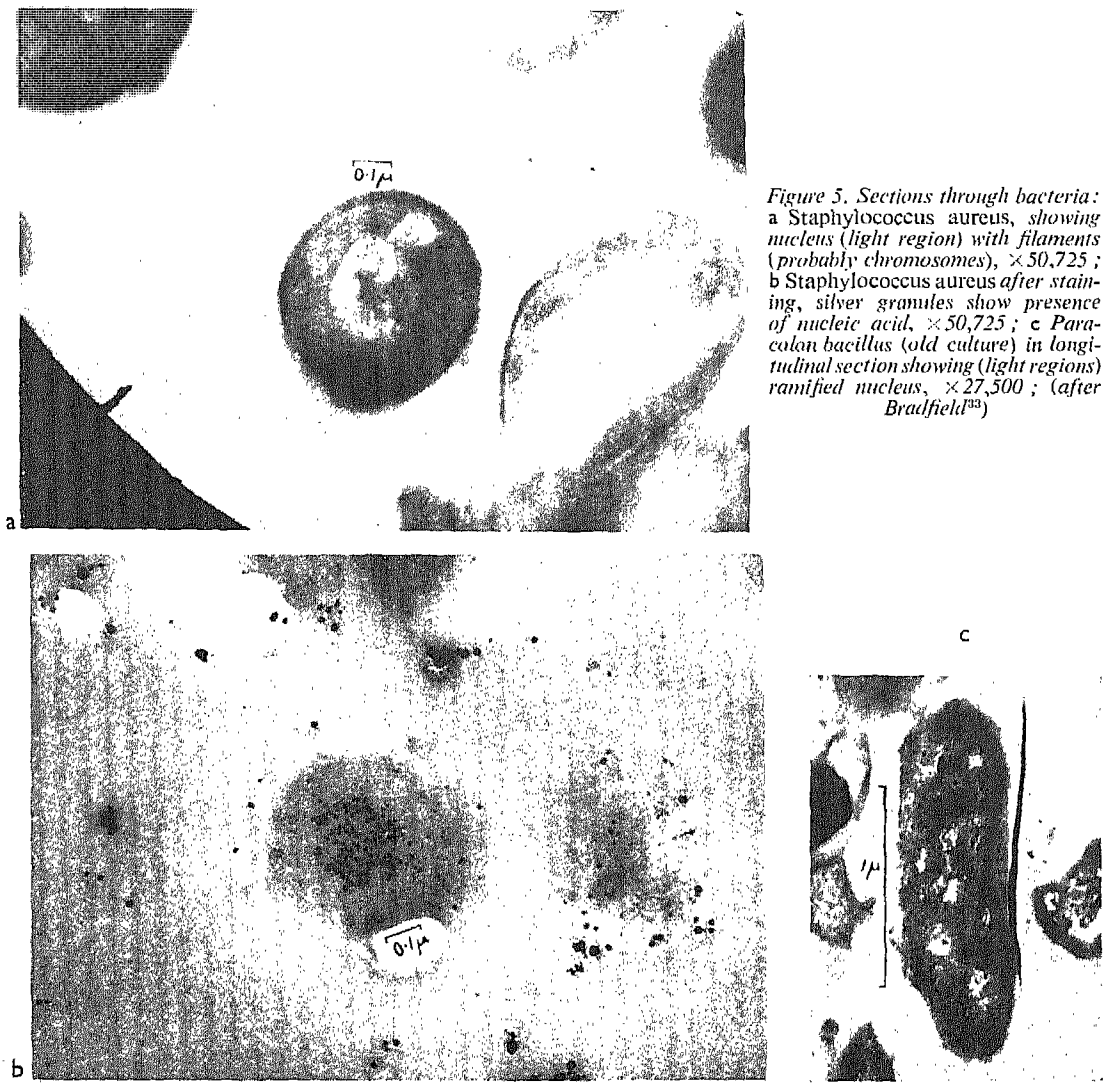

Figure 5. Sections through bacteria: a Staphylococcus aureus, *showing nucleus (light region) with filaments (probably chromosomes),* ×50,725; b Staphylococcus aureus *after staining, silver granules show presence of nucleic acid,* ×50,725; c *Paracolon bacillus (old culture) in longitudinal section showing (light regions) ramified nucleus,* ×27,500; *(after Bradfield*[33]*)*

than for optical microscopy. Electrons of 75 to 100kv will not give a clear picture of a layer of biological material which is more than about 0·25μ thick—or about the wavelength of ultraviolet light—compared with a minimum thickness of 1μ obtainable with a standard microtome. The mistaken idea was first pursued that much thinner sections could only be obtained by using a very high cutting speed, but it is now clear that almost any type of microtome advancing mechanism can be refined to give sections of 0·1μ or less. Some new types of microtome, specially designed for electron microscopy, will provide sections as thin as 0·02μ (200Å), so long as careful attention is paid to selection of embedding material and technique of cutting. Particularly important has been the work of A. J. HODGE, H. E. HUXLEY and D. SPIRO[24] at the Massachusetts Institute of Technology, of K. R. PORTER and J. BLUM[25] at the Rockefeller Institute for Medical Research, and of F. S. SJÖSTRAND[26] in the Karolinska Institute, Stockholm ; reference to much other work is given in these papers.

A sufficiently slow advance may be obtained either by gearing down a mechanical motion or by simple thermal expansion of a rod carrying the specimen, as first proposed by S. B. NEWMAN, E. BORYSKO and M. SWERDLOW[27]. A normal electric lamp placed a few centimetres from a metal rod 25 cm long will give an adequate expansion, of the order of 100Å/sec. Alternatively the rod may be cooled with ice or carbon dioxide, and cutting carried out as it warms up to room temperature again.

For embedding medium a hard plastic material is usually preferred, although sufficiently thin sections can also be obtained with the more orthodox media such as ester wax. The best method is to embed the tissue in a monomeric liquid, such as a mixture of butyl and methyl methacrylate, which is then polymerized under conditions such that the heat generated does not damage the specimen. A glass wedge is favoured for cutting, rather than the conventional microtome knife, following the demonstration by H. LATTA and J. F. HARTMANN[28] of its practicability for a plastic block. Various minor refinements are favoured by particular workers, but it seems to be generally agreed now that cooling of the block is not necessary ; very thin sections can be cut at room temperature so long as the rate of cutting is kept low—a few seconds per stroke.

Examples of the results obtainable from thin sections are shown in *Figures 5* and *6*, the latter taken from a comprehensive survey by J. R. G. BRADFIELD of recent work on the structure of cytoplasm[29]. Sections through bacteria can be cut without difficulty, and *Figure 5* compares the internal structure of a coccus and of a rod bacterium as thus revealed. It seems clear that, in contrast to much early speculation, bacteria do indeed possess nuclei and that these are normally more electron transparent than the rest of the cytoplasm. The work of C. F. ROBINOW, reported in the previous survey[1], has thus been substantiated as against the view that the dense bodies often observed in bacteria are the nuclei.

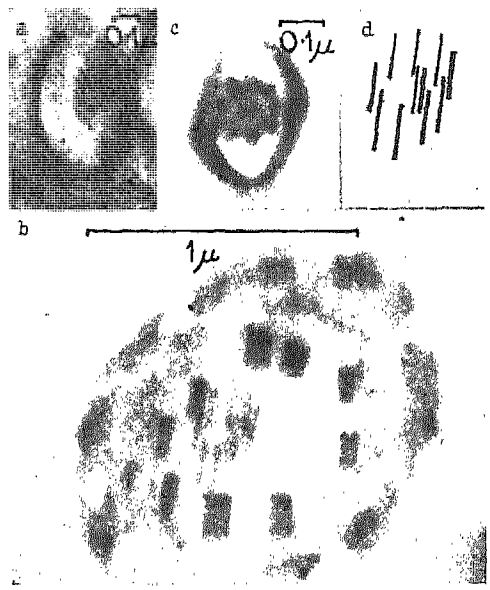

Figure 6. Sections through sperms showing rings of nine fibrils around two axial threads : a *sea urchin sperm, with one ring of nine fibrils, ×33,750 ;* b *bull sperm, near head, with two rings of nine, ×36,000;* c *bull sperm, near end of tail, with one ring of nine or possibly two rings fused together, ×60,000;* d *bull sperm, diagrammatic representation of* b *(after Bradfield[29])*

The nature and function of these dense bodies have been the subject of much investigation, so far without conclusion. The energy producing functions of mitochondria have been ascribed to them[30], but this view was recently contested, especially by A. WINKLER[31]. In *Mycobacterium tuberculosis* they may possibly partake of the nature of spores, since they vary greatly in density and in number during the growth cycle[32].

For solving this type of problem it is important that methods of differential staining for electron microscopy, using heavy elements, should be developed in analogy with normal microscopy, in order to distinguish the biochemical nature of particular regions of cells and tissues. A beginning

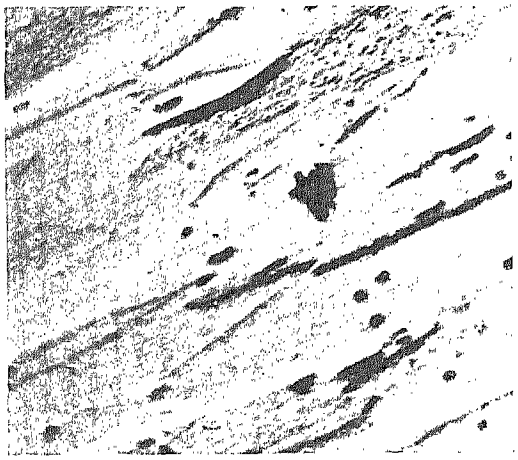

Figure 7. Reflection electron micrograph of etched surface of Armco *iron, showing grain boundaries and debris—magnification × 133 in direction of illumination and × 1330 across it (after Cosslett and Jones[39])*

in this direction has been made by J. R. G. BRADFIELD[33], who introduces silver into the Feulgen reaction for deoxyribonucleic acid so that the nucleus will appear electron dense (*Figure 5*b), and by G. BAHR[34] with other methods for nucleic acids and SH-groups.

It has only been possible to mention a few of the more promising recent advances in biological techniques in this survey. Many other methods are being developed, such as the selective digestion of organisms and tissues. Detailed references are given in the bibliographies of electron microscopy[35,36].

Metallurgical techniques—Until recently the electron microscopy of metal specimens has been dependent on the use of replica methods to provide a specimen for viewing in transmission. Some important advances have been made in this art, but in the past few years the method of reflection microscopy has been fully explored and its usefulness demonstrated, with the result that some commercial instruments are now being fitted with reflection adaptors. By this means it is possible to examine the surface relief of a specimen directly, with the electron beam at grazing incidence on it, and in conditions where replica methods would be impracticable, such as high temperature treatment and mechanical or chemical action.

The reflection method was first investigated by B. VON BORRIES[37] and others in 1940–41, when they examined some typical metallurgical specimens in an adapted Siemens microscope. With the increasing importance of metallurgical applications, the method was revived and developed. In some cases existing microscopes have been adapted and in others special reflection models were built[38–40] in order to provide for greater flexibility of action and the admission of specimens of a size of interest in practical metallurgy. The optimum conditions for examining different types of surface have now been established, especially by M. E. HAINE and W. HIRST[41] and J. W. MENTER[42]. A wide range of applications has been explored by Menter, as reported at the London Conference, and high temperature operation successfully demonstrated by D. JONES[43].

A typical reflection micrograph is given in *Figure 7*, which illustrates the prominence of the surface relief when the beam falls on the surface at an angle of a few degrees. The resulting picture is naturally foreshortened in the direction of viewing, since the best definition is obtained when the viewing angle is approximately equal to the grazing angle. For investigating very small changes in surface elevation, angles of less than 1° have been employed[41]. It is therefore necessary to observe care in comparing the results with optical micrographs taken at normal incidence.

The reflection method having become properly established, there can be little doubt that it will find many applications, and not only in metallurgy, since insulating surfaces can also be investigated so long as they are first coated with a conducting layer of metal by evaporation. But the resolving power obtainable is very much worse than that of transmission methods, owing to the large energy losses that occur in reflection. A large part of the reflected beam entering the objective lens has suffered energy loss of the order of 100v, so that the initial monochromaticism has been lost. The high chromatic aberration of the lens then gives a resolution of no better than 200 to 400Å, according to the aperture employed. Whilst this is some ten times better than the limit of the optical microscope, it is some ten times worse than a good transmission electron microscope. By reducing the aperture, the aberration could be reduced below 100Å, but only at the cost of also reducing the image intensity below that which will give a visible image on the screen at any useful magnification. Only the development of an achromatic lens, as already stated, or of a velocity filter that will reject electrons which have suffered more than a few volts energy loss, can remove this limitation. In the meanwhile there is ample scope for exploration with the reflection method in the region just outside the optical limit.

The main methods of preparing thin film replicas of surfaces have been described[19] by Drummond.

Since then some advances have been made in replicating special types of surface, such as wood[44-46] and ice[17]. But the most important step has been the discovery of a method, of wide applicability, that has a much better inherent resolution than any other so far used, in the carbon film process due to D. E. BRADLEY[18]. By passing a heavy current between two carbon rods that are in light point contact, in a vacuum, carbon is evaporated and may be collected on a neighbouring surface. In this way a film may be obtained which is extremely tough, yet thinner than 100Å; this is useful for supporting very thin specimens such as 200Å sections. If the carbon is collected on the surface of a metal, a replica of it may be obtained directly, but it is not always possible to detach the film without etching away the metal. It is generally preferable to make a first replica in the usual *Formvar* or other plastic film, which can readily be removed from the metal without damage, and then to evaporate carbon on to this to obtain a second replica. The latter is then a positive impression of the surface, rendering interpretation rather easier than when using a single-stage, negative replica.

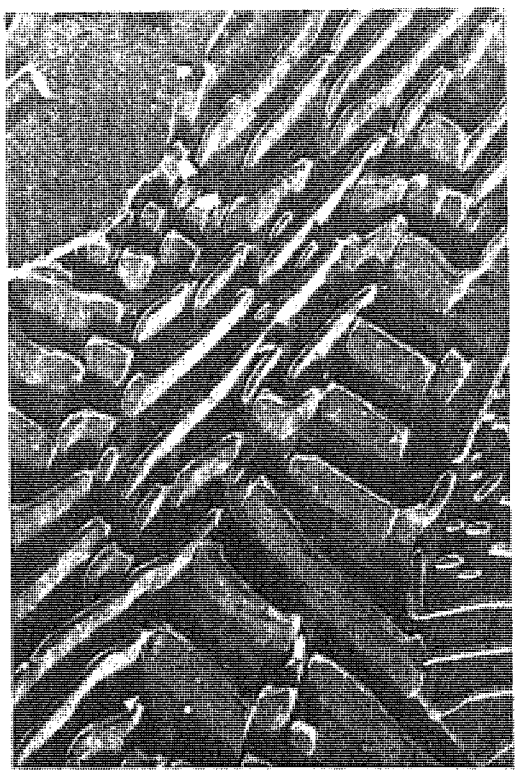

Figure 8. Carbon replica of pearlite structure, ×6666 (after Bradley[61])

The resulting replicas show extremely high resolution, even when obtained from a first replica of *Formvar* or nitrocellulose; it is clear now that the much poorer resolution given by the latter is due to body structure in the material, whereas the surface contains a very exact impression of the original surface. The carbon film itself appears to show no structure down to the very best resolution so far used to examine it—certainly not of the order of 20Å. Thus, at last there is available an easily used replica method which will reproduce surface detail as fine as the electron microscope can reveal. It is already finding applications to metallurgical problems, as contributions to the Conference showed. A typical result is given in *Figure 8*.

APPLICATIONS OF THE ELECTRON MICROSCOPE

The wide range of uses of the electron microscope in scientific and technical research is indicated by a list of the topics which appeared in the Conference programme. In virology, the internal structure of animal viruses, the multiplication of insect viruses and the growth of plant viruses were discussed. In bacteriology, the structures of several species have been studied—especially by thin sectioning technique—and there were two papers on tuberculosis. The internal structure of cells was the theme of a dozen papers, mostly using thin sectioning methods and ranging from liver, pancreas, nerve, thyroid, epithelial and adrenal cortical cells to the more homely yeast and aspidistra. Another session was devoted to body and cell wall structure: animal epidermis, insect cuticle, wood and paper; yet another to fibrillar structures: muscle, collagen, cellulose and silk. A special symposium was concerned with recent work on the microanatomy of the minute 'tails' of micro-organisms, in the form of cilia, flagella and similar processes: *Figure 6* indicates the remarkably complex structure within a sperm tail, the role of which in motility is not yet clear.

In metallurgy, papers dealt with heat resistant alloys, graphitization, tempering of alloy steels, the austenitic transformation, and pearlite structure. In the non-ferrous field, there were studies of tungsten carbide, aluminium-copper and related alloys and gold. In more fundamental metal physics, investigations were reported on recrystallization, solid state transformations, and the mechanism of fracture and of slip in plastic deformation.

Industrial and chemical applications also attracted a dozen papers. In the chemical field were studies of crystal nucleation and growth, surface reactions of metals, hydration of silicates and properties of clay colloids. The more industrial applications concerned silicone rubber, paper sizing, surface

structure of diamond, nylon fibres, plastics and starch. Since about half the electron microscopes in the world are situated in industrial laboratories, there is undoubtedly a great deal of work in progress in applied science and technology which is not being published.

It is evident that no detailed account of particular applications could be given in a survey such as this. The Conference Handbook gave an abstract of each paper and the name and working place of the author. A survey, with 188 references, of recent work has been compiled by Swerdlow[36] and a review of biological advances by Bradfield[29]. Comprehensive and up-to-date textbooks of electron microscopy are also now available[19,50], and abstracting schemes are being operated by the New York Society of Electron Microscopists, the Royal Microscopical Society in Britain and the German electron microscope society. As in other branches of science, it is becoming difficult to keep pace with the volume of publication—the Japanese have even started a Journal of Electron Microscopy, with an English as well as a Japanese edition. It will be one of the main tasks of the new Joint Commission to try to bring some order into this field, and especially to unify the efforts of the several national societies.

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DEVELOPMENT OF REFLECTING MICROSCOPES

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Although the reflecting microscope has been known since Newton's day, it is only relatively recently that it has become an important instrument of research. The reflecting objective has a number of important advantages : it can be made achromatic for a very wide range of wavelengths and it may be designed with a large numerical aperture. In this paper the author traces the history of the reflecting miscroscope and describes some of the important features of current reflecting objective design.

TWO WELL DEFINED periods of development may be recognized in the history of the reflecting microscope. Between 1672 and 1850 both reflecting and refracting objectives were improved in an attempt to increase the performance of the objectives then in use. During that time the reflector was not used for its special characteristics but because of its general superiority over contemporary refractors. This was due mainly to the lack of optically homogeneous glass and the difficulty of correcting chromatic and spherical aberration in the simple lenses employed in the construction of the refractors. As a result of the great improvements in the refracting objective, which followed the introduction of the achromatic lens in 1824, the reflecting microscope soon became obsolete.

The second stage of development started in 1931 and is still continuing. It is due almost entirely to the complete achromatism of the reflector, its long working distance and its ability to transmit a wide range of wavelengths. Whilst great improvements have been made in the design of reflecting objectives during the past two decades, it is instructive to consider the progress made by the pioneers of optics during the first stage of development.

Sir ISAAC NEWTON described what was probably the first reflecting microscope in a letter to H. OLDENBURG, the secretary of the Royal Society, in 1672 ; it consisted of a concave ellipsoidal mirror and a diagonal flat. Twenty years previously Newton had described his own reflecting telescope and he may well have realized that, if light be sent backwards through a telescope objective, it becomes a micro-objective of long focal length. In the same way R. BARKER[1] (1736) and B. MARTIN[2] (1759) based the design of low numerical aperture reflecting objectives upon the Gregorian telescope. At about the same time R. SMITH[3] (1738) described a number of single- and double-mirror microscopes or ' compound reflecting engiscopes ' as he called them. The two-mirror objectives were of the Cassegrain type and a commercially made objective, designed by Smith and bearing the inscription S. J. Rienks Leyden 1826, is still in existence. Extracts from Smith's remarkable book give some idea of the reasoning which led him to his designs ; for example :

> ' This put me upon contriving a microscope with two reflecting spherical surfaces of any size so proportional to each other that the aberrations of the rays caused by the first reflection shall be perfectly corrected by the second ; and by consequence that the last image of the object, from which the rays diverge upon the eye glass shall be as perfectly free from aberrations as the theory of the aberrations is perfect—I found that the colours of objects in a reflecting microscope appeared much more beautiful and natural than in double refracting microscopes of the best sort, their proper colours being free from the mixture of other colours arising in refracting microscopes from the different refrangibility of the rays.'

The Smith-type reflecting microscope continued in use until the beginning of the nineteenth century,

when G. B. AMICI constructed a reflecting microobjective in which the object was placed at one focus of an ellipsoidal mirror and the image was formed at the other. The numerical aperture of a typical Amici objective was 0·2 and, despite the large amount of coma which must have been present, it compared favourably with any refractor then in use.

CUTHBERT, an English instrument maker, manufactured the Amici-type instrument using a series of interchangeable ellipsoidal mirrors for different magnifications. The shortest focal length mirror had a numerical aperture of 0·46 and this represented the peak of achievement in the first stage of development.

PROPERTIES OF REFLECTING OBJECTIVES

Before reviewing the progress that has been made in recent years in the design of reflecting microscopes it is profitable to consider briefly the general properties of reflecting objectives. A simple Schwarzschild-type reflecting objective (*Figure 1*) consists of two mirrors—one concave (M_1) and the other convex (M_2). The mirror M_1 produces an image of the object at I' and this image is then magnified by the mirror M_2 to produce an enlarged image at I. Due to the diffraction suffered by light passing through the objective, a point object gives rise to a disk of light at I surrounded by a series of rings of light of decreasing intensity. This disk of light (the Airy disk) has a radius of $0{\cdot}61\lambda$/N.A. (for no central obstruction) where λ is the wavelength of the light used and N.A. is the numerical aperture of the objective. The numerical aperture is defined by N.A.$=\mu\sin\theta$ where μ is the refractive index of the material in which the object is immersed and θ the semi-angle of the cone of light entering the objective.

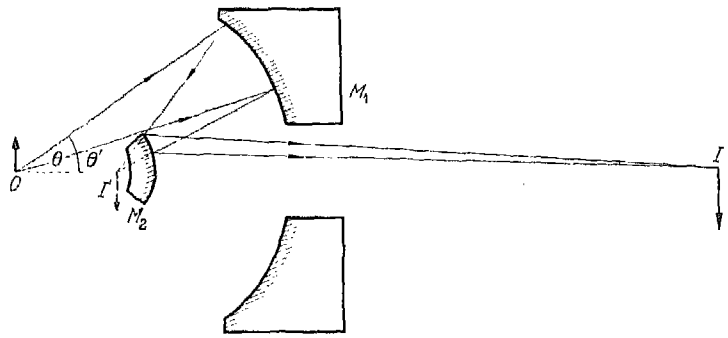

Figure 1. Path of light through reflecting objective

The size of the disk determines the amount of detail which can be detected in the object and two objects are said to be resolved when their geometrical images are separated by a distance $\rho=0{\cdot}61\lambda$/N.A. To decrease ρ it is necessary to increase the N.A. or to decrease the wavelength of the light used. In attempting to increase the angle θ, spherical aberration ϕ soon becomes the limiting factor since, for a bispherical reflecting objective, $\phi \propto$ (N.A.)^8.

Unfortunately the small mirror in a reflecting objective obstructs light which would otherwise fall on the concave mirror and this has the effect of disturbing the light intensity in the diffraction pattern in such a way as to decrease the light intensity in the Airy disk and to increase the light intensity in the first and other diffraction rings. The distribution of light in the diffraction pattern is more distorted as the central obstruction (C.O.$=100\ \sin\theta'/\sin\theta$) is increased[4–6] and when the central obstruction has a value of 55 per cent, less than 50 per cent of the light is in the Airy disk compared with 87 per cent for an unobstructed aperture. Astronomers were just able to detect the decrease in performance of a reflecting telescope[7] when the central obstruction was 30 per cent or greater and it has been the aim of most microscope designers to restrict the central obstruction to this limit. However, it will be seen later that for some purposes it is convenient to use larger values.

Between 1850 and 1930 interest in reflection optics centred on the construction of large astronomical telescopes and in 1905 K. SCHWARZSCHILD[8] developed a general theory of aplanatic mirror pairs. Little attention was paid to this classical work until many of the results were rediscovered independently by H. CHRÉTIEN[9] in 1922. At about this time[10] microscopy was extended to shorter wavelengths as a means of increasing the resolving power of the microscope and of studying the cytology of tissue cells by using the differential absorption of the nucleus. The techniques then available, involving quartz monochromatic refracting objectives, were slow and difficult to use, and it was for these reasons that the reflecting microscope entered into a new phase of development.

The reflecting microscopes constructed since 1931 can be divided into four main groups and for the sake of simplicity each of these will be considered separately although in fact they were not developed independently. The four groups are: (*1*) single-mirror objectives, (*2*) two and three-mirror objectives, (*3*) catadioptric objectives, (*4*) solid objectives.

Single-mirror objectives—In 1934 B. K. JOHNSON[11] described the first reflecting objective to be used as an ultraviolet microscope. The spherical concave mirror was of the 'mangin' type *i.e.* a back-aluminized quartz meniscus lens (*Figure 2*). Light from the object was introduced into the system by an inclined plate of quartz and after reflection at the mirror it was brought to a focus in the image space by a single plano-convex lens which was refocused for each wavelength. The objective of numerical aperture 0·5 could be used in the vacuum ultraviolet.

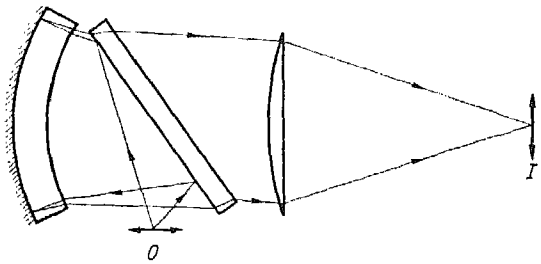

Figure 2. Single-mirror reflecting objective with correcting lens

By 1938 the field of view of astronomical telescopes had been increased as a result of the successful development of the Schmidt corrector plate, and in 1938 E. H. LINFOOT[12] applied the same principles to the construction of an ultraviolet micro-objective of 0·54 N.A. The object was placed inside the focus of a spherical mirror which had a quartz corrector plate at its centre of curvature (*Figure 3*). The inaccessibility of the object point was a great disadvantage of this design.

The availability of synthetic optical crystals enabled B. K. JOHNSON[13] to improve his microscope. He replaced the single quartz lens with a lithium fluoride and quartz doublet and the 'mangin' mirror with a parabolic mirror. The resulting objective was corrected for all wavelengths between 6000 and 2600Å.

D. D. MAKSUTOV[14] considered in some detail the theory of meniscus lens correcting plates and in 1949 B. K. JOHNSON[15] further improved his ultraviolet objective by reverting to a back-aluminized fused quartz meniscus lens as the reflecting component. The reduction of the angle of incidence on the mirror inside the quartz enabled the numerical aperture to be increased to 0·84. With the addition of a normal incidence achromatic immersion lens the N.A. was increased to 1·27.

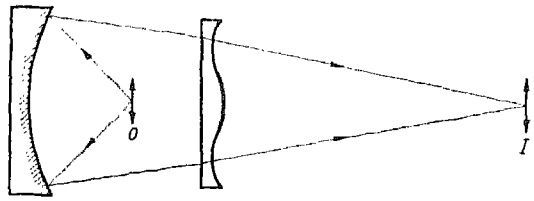

Figure 3. Single-mirror objective with Schmidt corrector plate

By comparison the next reflecting objective is extremely simple. It was designed by A. BOUWERS[16] in an attempt to provide an inexpensive teaching microscope of low numerical aperture (0·2). The object was placed at the focus of a concave mirror and in front of a hole in an inclined plane mirror (*Figure 4*). Light from the object was reflected by the concave mirror on to the plane mirror and then into an inclined eyepiece.

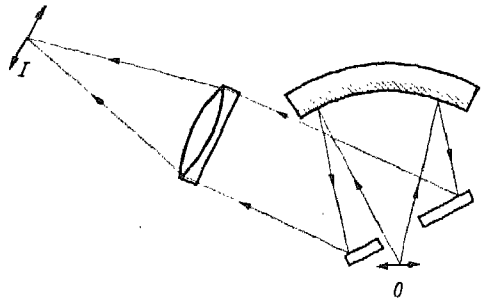

Figure 4. Simple reflecting objective of low numerical aperture

By 1949 several Schwarzschild-type reflecting objectives had been described and the inherent long working distance of this design had attracted the attention of metallurgists who were interested in examining the surface of metals contained in a furnace. It was for this reason that J. DYSON[17] constructed a 0·5 N.A. long working distance optical system with unit magnification. The object was placed at the centre of curvature of a concave mirror which had a hole at its pole; a weak lens between the object and the mirror acted as a corrector plate and served to reflect the image into a plane behind the concave mirror where it could be examined by a conventional microscope objective

(*Figure 5*). A special immersion system (N.A. 0·75) capable of working through 0·25 in. glass has been designed along these lines to facilitate the examination of photographic emulsions exposed to ionizing radiations.

The final objective in this group was constructed by A. ELLIOTT, E. J. AMBROSE and R. B. TEMPLE[18] for use in the near infra-red region at a magnification of ×5. It consisted of an off-axis paraboloid figured by evaporating selenium through a baffle on to a spherical mirror. This objective, used with an infra-red spectrometer and with polarized infra-red radiation, gave results which were of importance in the elucidation of the structure of synthetic[19] polypeptides and of fibrous[20] proteins.

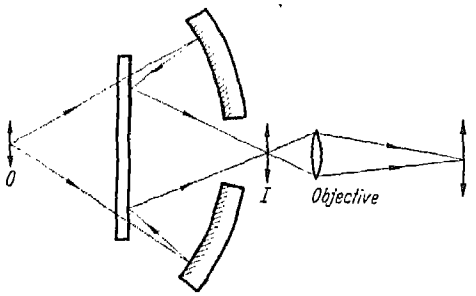

Figure 5. Long working distance objective

In modern research work a particular micro-objective is often designed for a specific purpose; this is particularly true of the single-mirror objectives described in this section and consequently they have not been widely used. A further factor which has tended to restrict the application of these objectives is that they cannot normally be used on a conventional microscope stand.

Two-mirror objectives—Smith rightly pointed out, over two centuries ago, that the aberration terms from a concave and a convex mirror are of opposite sign and the corrections necessary to produce a Schwarzschild objective are less than with any other combination of mirrors. It is for this reason that two-mirror objectives have been constructed in such large numbers in recent years.

The first bispherical Schwarzschild reflecting microscope was described in 1941 by S. A. HERSHGORIN, P. D. RADCHENKO and E. M. BRUMBERG[21]. The primary concave mirror had an axial hole through which the light passed from the small convex mirror to form the image (*Figure 1*). The N.A. was 0·5 and the mirrors, it seems, were monocentric *i.e.* they were concentric. Brumberg was the first to apply such an objective to the study of biological material in the ultraviolet region and he developed the three-colour ultraviolet translation microscope[22] in which three photographic negatives of the same specimen, taken at three different ultraviolet wavelengths, are projected—or reproduced on colour film—through three visual filters. The result is a coloured image in which changes of colour represent changes in ultraviolet absorption. This technique has since been perfected by E. H. LAND and his co-workers[23] using a 0·4 N.A. reflector designed by Grey (see below).

C. R. BURCH[24] in 1943 explained how he was induced by the work of Brumberg to make his own reflecting objectives. He showed that an aplanatic monocentric reflecting objective can be made from spherical mirrors but the price that has to be paid for this simplification is a large central obstruction (45 per cent). To reduce the central obstruction to 14 per cent whilst preserving anastigmatism it is necessary to use aspherical mirrors although a semi-aplanat of 0·65 N.A. can be constructed by using only one aspherical surface. Burch also expressed the opinion that two-mirror systems would eventually be abandoned in favour of solid reflecting objectives of the type to be described later. Further papers[25,26] by Burch published in 1945 and 1947 described more fully the design and construction of his elegant objectives which are now being manufactured in limited quantities. A glass immersion lens added to one of his objectives increased the N.A. to 0·98 and Burch showed that by the correct choice of the radius of curvature of this lens it was possible to reduce the curvature of field. Without doubt these objectives are the most highly developed to date although the production of aspherical mirrors to the degree of accuracy required by the design is a difficult and costly process. The final polishing of the large mirror is performed by hand, and this results in a 'cobbled' surface which may give rise to undesirable diffraction effects in the ultraviolet region of the spectrum. Because of this, doubts have been expressed[27] regarding the use of a Burch microscope for ultraviolet microspectrography, but it remains to be seen whether these doubts have any foundation. The Burch microscope has been used in conjunction with an ultraviolet[28] spectograph and an infra-red spectrometer[29] although for the latter purpose it appears to be a luxury since bispherical objectives are quite adequate.

The only other two-mirror aspherical microscope to be described is a 0·6 N.A. objective constructed by D. L. WOOD[30] in 1950. This Burch-type objective was used with an infra-red spectrometer at a magnification of ×65 and the results obtained do not seem to have justified the construction and use of aspherical mirrors. The cone of light leaving the

microscope at a magnification of ×65 was insufficient to fill the aperture of the spectrometer and the performance of the system might have been improved by reducing the magnification of the objective to about ×10.

The calculations of Burch were used by A. H. BENNETT, D. L. WOERNLEY and A. J. KAVANAGH[31] to design a bispherical ultraviolet objective of N.A. 0·3 which they used for experiments in ultraviolet phase microscopy. The resulting pictures were extremely difficult to interpret in terms of absorption and phase effects. Land and his collaborators in 1949 used an objective of slightly superior performance (N.A. 0·4 designed by Grey and manufactured by the Polaroid Corporation) in their development of the Brumberg three-colour translation microscope[23]. Later in the same year R. C. GORE[32] used a similar objective and condenser in an infra-red microspectrometer to measure the absorption spectrum of small crystals. Early in 1950 E. R. BLOUT, G. R. BIRD and D. S. GREY[33] used a similar objective in a more efficient microspectrometer. They discussed the improvements which might be effected by increasing the N.A. to 0·63 and by adding a thallium bromo-iodide immersion lens to increase the N.A. to 1·5. The use of these objectives in the infra-red was described in later papers[34,35] by the same authors. They came to the conclusion that the light losses by reflection when an immersion lens is added to an infra-red reflecting objective outweigh the advantages of the increased aperture. Furthermore, no suitable immersion liquid has yet been found for any likely lens material.

To facilitate the photography by ultraviolet light of living mitotic tissue culture cells W. E. SEEDS and M. H. F. WILKINS[36] constructed a monocentric objective of 0·5 N.A. and central obstruction 47 per cent. When the aberrations of this objective were measured with a microscope interferometer they were smaller than those predicted by the Burch-Schwarzschild theory, and this prompted K. P. NORRIS, W. E. SEEDS and M. H. F. WILKINS[37] to investigate how far it was profitable to deviate from the monocentric design. They described a series of objectives of various numerical apertures and central obstructions in which the convex mirror was mounted on a set of kinematic slides to enable it to be positioned accurately relative to the concave mirror. Having the adjustments gives the advantage that spherical aberration due to variation in the thickness of cover slips and changes in tube length can be corrected.

Previously a monocentric objective of N.A. 0·5 had been scaled up in size by D. W. DEWHIRST and M. J. OLNEY[38] to increase the working distance so that they could examine the surface of metals contained in a furnace.

R. D. B. FRASER[39] in 1950 described what was probably the first infra-red microspectrometer in which an attempt was made to obtain the maximum transference of energy from the objective to the spectrometer and this used a monocentric objective of N.A. 0·8. Used at a magnification of ×11 all the energy which passed through the specimen was used to fill the aperture of the spectrometer. A numerical aperture of 0·8 can be used in the infra-red only because of the long wavelength of the radiation[40]. In the ultraviolet it has been shown[41] that the numerical aperture must be restricted to 0·6.

A new approach to the problem of constructing a high aperture ultraviolet objective was made by K. P. NORRIS and M. H. F. WILKINS[42] who added a quartz Amici lens to a reflecting objective of 0·6 N.A. The resulting objective which had a N.A. 1·3 was polychromatic and the Amici lens had to be refocused for changes of wavelength. By reducing the N.A. to 1·25 the residual axial aberration for all wavelengths between 2650 and 5460Å was less than $\lambda/10$.

A paper by W. H. STEEL[43] in 1951 discussed the theory of Schwarzschild pairs. He showed, as Grey had done previously, that low central obstruction designs are monocentric and have a large coma-free field at low magnification. To emphasize this point he designed a three-mirror reflecting objective in which a further concave mirror was used to form a magnified secondary image of the first image formed at low magnification. He concluded that non-concentric designs such as those described by Norris and his co-workers are not as promising as three-mirror objectives and are too difficult to adjust. However, the present author maintains that such objectives, when correctly used, are capable of yielding valuable results.

S. MIYATA, S. YANAGAWA and S. NOMA[44] constructed objectives of 42 and 36 per cent central obstruction with a normal incidence glycerol immersion lens to increase the N.A. to 1·0. The point of interest in the Japanese paper is that the authors made their objective aplanatic (free from coma and spherical aberration) by evaporating zinc sulphide through a baffle on to the concave mirror until the right degree of asphericity was obtained. For convenience they mounted the convex mirror on the back of the immersion lens.

Apart from a paper by B. K. JOHNSON[45], who used two bispherical reflecting objectives of N.A. 0·66 and 0·25 for ultraviolet microscopy in the region of wavelengths 2000 to 1000Å, all the remaining papers

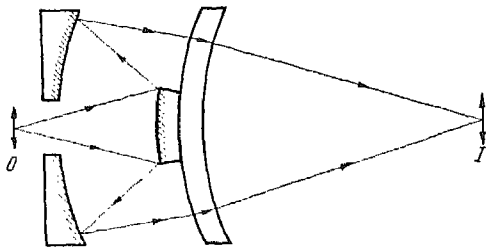

Figure 6. Newtonian objective with meniscus correcting lens

on two-mirror objectives are concerned with infra-red applications.

An objective and condenser of N.A. 0·56 and C.O. 47 per cent were used by R. M. BADGER and R. NEWMAN[46] in the construction of an ingenious infra-red micro-illuminator which they used at low temperatures. Subsequently A. R. H. COLE and R. N. JONES[47] constructed a reflecting objective of N.A. 0·5 and C.O. 30 per cent, which was designed for working at a magnification of ×10 with an infra-red spectrometer. A further improvement was reported in 1953 by V. J. COATES, A. OFFNER and E. H. SEIGLER[48] who described the construction of an objective of 0·75 N.A. which they too used for infra-red microspectroscopy.

Of two-mirror reflecting microscopes it can be said that they have already made a real contribution to spectroscopy and to science in general. With the aid of a reflecting objective, infra-red spectra have been obtained from samples weighing as little as 1 μg. The present day trend is towards dry objectives of high numerical aperture since the aberrations are of secondary importance to the light gathering power. In the visible region of the spectrum the main use of reflecting objectives appears to be for metallurgical purposes where the long working distance is a great advantage. For ultraviolet microscopy and microspectrography a bispherical objective of 0·6 N.A. can be used, but the advantages of solid reflectors are such that they may eventually replace the two-mirror designs.

Catadioptric objectives—Objectives in this class have been studied in great detail by Grey, although other authors have made valuable contributions. E. M. BRUMBERG[49] in 1943 described the first bispherical micro-objective which used a correcting lens. The chromatic aberration produced by the cover slip was corrected by a thick meniscus lens in the image space. A similar device was used by Norris, Seeds and Wilkins for their ultraviolet objectives[37]. The Maksutov meniscus lens mentioned previously was used[16] by Bouwers to increase the N.A. of an objective to 0·6. Light from the object, which was placed just behind a concave mirror containing an axial hole, was reflected at the secondary convex mirror on to the concave mirror; it then passed through the meniscus correcting plate to form the primary image (*Figure 6*). Similar objectives with a plane mirror in place of the convex mirror and with more correcting plates to increase the N.A. to 1·0 were designed by D. S. GREY and P. H. LEE[50]. These objectives were eventually discarded in favour of catadioptric Schwarzschild objectives[51] since the latter offered greater possibilities for reducing the central obstruction. The aperture of a 0·4 N.A. reflector was increased by placing lenses between the object and the secondary mirror. The image from the mirrors was formed at low magnification to reduce the central obstruction while retaining monocentricity; more lenses were placed between the secondary mirror and the image to increase the magnification and to improve the correction (*Figure 7*).

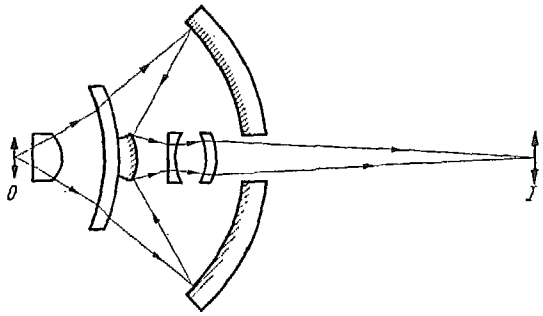

Figure 7. Catadioptric Schwarzschild objective

In 1950 D. S. GREY[52] described a series of catadioptric Schwarzschild objectives computed for working in the ultraviolet region with N.A. up to 1·0 and C.O. ≃29 per cent. The catadioptric objectives are theoretically capable of greater correction than the simple reflectors, but the complexity resulting from the large number of optical surfaces involved and the difficulty of centering all the components places them at a slight disadvantage. For most ultraviolet purposes where a large field diameter is not necessary, the simple two-mirror objectives of N.A. 0·5 to 0·6 are adequate.

Solid objectives—The first reflecting objectives to be described in the second stage of development were the ingenious solid objectives of D. D. MAKSUTOV[53] who in 1932 showed that a sphere-cardioid combination is aplanatic. He constructed several solid objectives in which he skilfully utilized total internal reflection at a glass–air interface which afterwards allowed the light from the second mirror

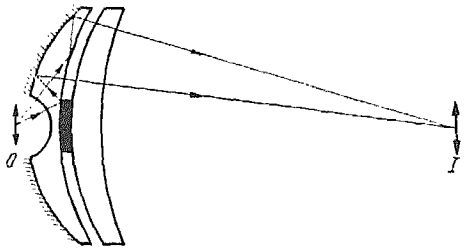

Figure 8. Solid reflecting objective

to pass at normal incidence (*Figure 8*). The majority of his designs involved two concave reflecting surfaces in the form of a thin meniscus lens. However, at least one was a solid Schwarzschild pair of 0·85 N.A. (*Figure 9*) in which the space between the mirrors was filled with glass, and the entrance and exit surfaces were so arranged as to have the object and image point at their centre of curvature. All these objectives which had a high central obstruction ($\simeq$ 50 per cent) were constructed in glass and by immersion of the object it was possible to increase the numerical aperture by a factor of approximately 1·5.

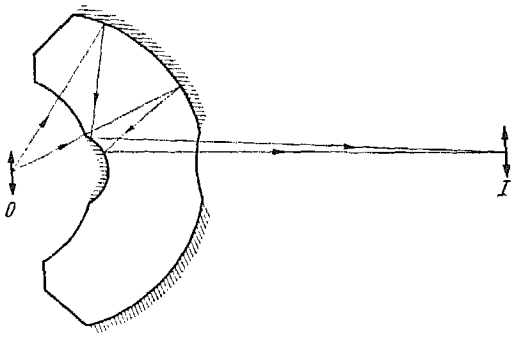

Figure 9. Solid Schwarzschild-type objective

In 1952 C. G. WYNNE[54] took out a British patent on a solid objective of 0·65 N.A. similar in design to the Maksutov-Schwarzschild objective. As he restricted himself to spherical surfaces it was necessary to add further lenses in the image space to correct the aberrations up to N.A. 0·75. R. L. DREW[55] constructed a solid achromatic flat-fielded semi-aplanatic objective of 0·6 N.A. from two highly aspherical concave surfaces (*Figure 10*). By immersing the object the N.A. was increased to 0·9. Drew discussed the possibility of making the objective in quartz so that it could be used in the ultraviolet region. K. P. NORRIS and M. H. F. WILKINS[56] overcame the difficulties of constructing a solid quartz Schwarzschild objective by making three quartz lenses with aluminium deposited on the appropriate surfaces. The lenses were then centered and placed in optical contact (*Figure 11*). Using water immersion the objective (N.A. 0·9, C.O. 35 per cent) was achromatic and could be used for ultraviolet micrography in the same way as an ordinary objective.

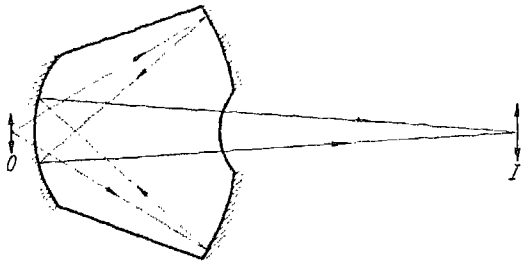

Figure 10. Solid semi-aplanatic objective

A. BOUWERS[57] had previously constructed solid glass objectives by cementing them together with Canada balsam. These objectives[58] were described in greater detail in 1952. The object of the work was to construct good and inexpensive apochromatic visible light objectives. The most complicated objective contains only five components and has a numerical aperture of 1·4 with a much larger field of view than a conventional oil immersion refracting objective. It seems as though the opinion expressed by Burch in 1934 will soon be realized and that this work on solid reflectors will eventually improve the standards of microscope construction.

CONCLUSIONS

Most of the instruments described here are based on spherical mirrors. The conclusions reached may well be modified by advances in the technique of making aspherical surfaces. Single-mirror objectives have been developed for specific purposes but they have not been widely applied in modern research because their construction does not normally permit them to be used on a conventional microscope stand. Catadioptric systems are theoretically

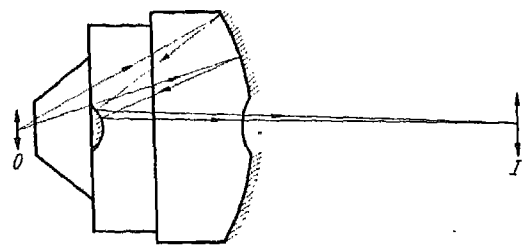

Figure 11. Solid quartz Schwarzschild objective

capable of greater correction than simple reflectors, but they are so complex that they should rightly be considered as refracting objectives.

The results so far achieved[59] with infra-red microscopes of numerical aperture 0·8, approximating to the monocentric design, indicate that little further improvement can be expected by adopting aspherical mirrors. It may however be an advantage to have an aspherical objective with a low central obstruction for measurements with polarized infra-red radiation.

Valuable results have been obtained with bispherical objectives of 0·6 N.A. in the ultraviolet region[60], but an objective of higher numerical aperture is desirable to fill the gap in the range of ultraviolet objectives now available. An aspherical objective of 0·9 N.A. (dry) would seem to satisfy the requirements of most users[61] since with immersion the N.A. could be increased to 1·3. For routine ultraviolet microscopy there is nothing better than a quartz solid reflecting objective; the availability of new synthetic ultraviolet transmitting crystals should enable the performance of existing objectives to be improved still further.

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APPLICATIONS OF THE REFLECTING MICROSCOPE

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In the preceding article in this series Dr K. P. Norris described the development of reflecting microscopes. In this paper Dr Roe shows how the particular properties of reflecting microscopes lead to their application in the ultraviolet and infra-red regions. She also discusses some of the applications to microspectroscopy in these spectral regions.

NEARLY three centuries have passed since the first simple reflecting microscope was constructed by Sir ISAAC NEWTON, and half a century ago K. SCHWARZSCHILD's work[1] on a two-mirror telescope objective led to the design of an aplanatic reflecting microscope of reasonable numerical aperture. Also at the beginning of this century, the range of observation with the refracting microscope was extended beyond the visible region to shorter wavelengths, when A. KÖHLER[2] and M. VON ROHR introduced quartz microscope lenses, acting on a suggestion which had been made even earlier by C. V. BOYS[3]. Important developments in the design of reflecting microscopes have taken place since these beginnings, and a few investigators have used quartz microscope optics to explore beyond the visible spectral region with excellent results. Recently, however, the significance of these advances has achieved wider recognition. Biologists, chemists and physicists are applying the reflecting microscope in a variety of special problems, particularly at those wavelengths in the ultraviolet and infra-red regions where the limitations of conventional refracting optics become apparent.

PARTICULAR APPLICATIONS AND DEFICIENCIES OF THE REFRACTING MICROSCOPE

The possible applications of the reflecting microscope are as diverse as those of the refracting instrument. Thus, it may be used with accessories for phase contrast and interference microscopy, with polarized and unpolarized radiation from the ultraviolet to the infra-red region of the spectrum. Addition of spectroscopic equipment allows its use for emission, fluorescence and absorption microspectroscopy in various spectral regions. A number of these applications are mentioned by R. BARER[4], J. R. LOOFBOUROW[5] and R. C. MELLORS[6] in useful review articles, and selected examples will be described below.

It is worth while noting some particular applications of the conventional refracting microscope and points at which its deficiencies appear, in order to understand the advantages of reflecting systems. In order to achieve high resolving power, it was natural that the use of shorter wavelength illumination should be explored, as is clear from the formula for resolving power (d), since

$$d = \frac{0{\cdot}61 \times \lambda}{\text{numerical aperture}_{\text{(objective)}}} \quad \ldots(1)$$

where λ is the wavelength. The production of quartz objectives made it possible to illuminate with ultraviolet radiation of wavelength down to the transmission limits of fused quartz (about 1850Å), with considerable gain in the detail observed. Ultraviolet microscopy, using objectives corrected usually for 2750Å, was applied to problems of particle size in paints and rubber pigments and in photographic emulsions, and to the structure of leather. The most outstanding results were obtained in metallurgy and biology, particularly by J. E. BARNARD with his collaborators F. V. WELCH and J. SMILES[3,7,8], by B. K. JOHNSON and L. C. MARTIN[9,10] in Great Britain, and by F. F. LUCAS[11,12], R. W. WYCKOFF[13,14] and their colleagues in the United States.

In these fields it became possible to take advantage of the differential absorption and reflection of ultraviolet radiation which often occurs so that, for example, contrast is obtained in unstained, unfixed biological material due to the varying ultraviolet absorption in different regions of the specimen. This had been recognized by the earliest investigators[2,3,9,12] who also noted that it would be possible to observe changes in fresh biological material under treatment with radiation or chemicals.

It was a short step from observing differential ultraviolet absorption to attempted measurement of these differences. Thus, if by means of the microscope, standard spectroscopic methods could be modified for the micro-scale, a potentially valuable tool was available for the examination of microspecimens and parts of larger ones. F. F. LUCAS[12], F. M. UBER[15], and P. A. COLE and F. S. BRACKETT[16] long ago applied this technique to biological material, but it was in the cytochemical investigations of T. CASPERSSON and his school[17,18] that it was used most widely. Selective absorption in the region 2400 to 3100Å in unstained cells is known to be due largely to protein and to purine- and pyrimidine-containing constituents (the nucleic acids or polynucleotides) and their complexes with other macromolecules, so that the object of these studies is the correlation of changes in these chemical compounds with the cell growth and function in which they play an important role. It is in their microspectroscopic applications, and particularly in the ultraviolet region, that the deficiencies of refracting objectives are seen most clearly.

First, the quartz objectives used were monochromats, designed and corrected for use at one wavelength (usually 2750Å or 2570Å). Their use at other wavelengths, even if refocused, leads to deterioration in image quality, the effect depending on the wavelength, the objective used and the size and other characteristics of the object[17,19].

A further result of absence of chromatic correction is the difficulty of focusing the image. The necessity of using a fluorescent eyepiece with ultraviolet radiation to locate the specimen and find an approximate focus, followed by a series of photographs to test the focus, limited early routine applications in metallurgical micrography[11]. In biological work the danger of radiation damage to fresh cells, while focusing, led to the use of visual dark ground examination by Barnard and the mechanical refinements of the Barnard–Beck microscope[7]. This was followed by the addition of phase contrast searching equipment to the modern ultraviolet refracting microscope[20,21]. By these means excellent photomicrographs have been obtained, but for microspectroscopy at more than one wavelength the procedure is particularly tedious.

The illuminating requirements of monochromat objectives impose further limitations if their resolving power is to be realized in micrography[16]. The half-intensity spectral band width of the illuminating monochromator must be kept within 2 to 10Å, depending on the objective and conditions of use, for satisfactory image quality. In microspectroscopy, this also means that absorption measurements are made by the point-by-point method at successive wavelengths, involving danger of influencing later results by radiation after-effects of earlier measurements, quite apart from inaccuracies arising under the first limitation mentioned earlier.

The limited spectral transmission of quartz (from about 1850Å in the ultraviolet to 3μ in the infra-red) prevents the use of these objectives in the vacuum ultraviolet where increased resolution as well as differential absorption and reflection effects can be of importance, and in the long-wavelength infra-red region of particular interest in the absorption spectroscopy of organic molecules.

Finally, in order to eliminate aberrations, a quartz monochromat may have as many as seven lenses, separated by air spaces, and when used in micrography (especially with vertical illumination) back-reflection from the surfaces of these lenses may impair image quality considerably, and much more seriously in ultraviolet than in visible light. For this reason, B. K. JOHNSON[22] early constructed a 'reflecting microscope' employing a back-coated lens mirror with one auxiliary lens, in place of the usual refracting objective. He was able to use this for the micrography of alloys down to 1990Å.

ADVANTAGES AND LIMITATIONS OF REFLECTING MICROSCOPES

The advantages of the reflecting microscope, compared with a refracting system, can now be stated fairly simply. Its most important property is achromatism, so that the first three difficulties listed above, due to the chromatic aberrations in a refracting system, are overcome.

Dependence on reflection rather than transmission extends the useful spectral range over which the microscope may be applied. Aluminized surfaces have high reflectivity for this purpose, from about 1000Å in the vacuum ultraviolet region to the long-wavelength infra-red, although a gold surface has also been used for infra-red microspectroscopy on account of its good reflectivity in this region and greater durability.

Reduction in glare through reduction in the number of optical surfaces greatly improves image definition at all wavelengths as well as improving the ultraviolet micrography of opaque objects and enabling such work to be extended to even shorter wavelengths.

Finally, an important feature of reflecting systems is their long working distance. In the Burch[23] microscope of numerical aperture (N.A.) 0·65, the distance from the mounting of the small mirror to the focused specimen is 12 or 13 mm, to be compared with a working distance of about 0·5 mm for a refracting objective of equivalent numerical aperture. This makes possible operations such as the building of accessory apparatus on the microscope stage, micromanipulation of bulky objects, variation of specimen temperature, observation through considerable thicknesses of material *etc*—any of which may be important in special applications.

Of the various designs[24] of reflecting microscopes constructed during the last fifteen years, each can be a valuable tool in particular investigations. In fact, it is clear that the conditions of application should determine the design of instrument to be used, and only the instrument of optimum design, with aberrations within accepted tolerances, should be expected to perform a variety of functions. Recently there has been considerable discussion of this subject[25–29], emphasizing mainly the effects of aberrations on effective field diameter in mirror-pair objectives. Briefly, it is known that if the monocentric spherical mirror-pair is to remain aplanatic, *i.e.* spherically corrected and coma-free, its numerical aperture is limited to about 0·5. While the numerical aperture may be increased and spherical aberration still restricted within its tolerance by departing from the monocentric condition, the coma introduced reduces field diameter. It is calculated[28] that for such an objective, of 0·65 numerical aperture, the combined effects of coma, spherical aberration, and central obstruction limit the permissible object field diameter to 10μ or less at 2500Å; and it has been suggested that these be called 'small-field objectives'[25]. They have been developed mainly for microspectroscopy and such a field, *i.e.* sufficient to view a single rather small cell, is often all that is required. Within this field the familiar Airy diffraction pattern given by a 'pin-hole' object will still appear with energy removed from the central region, (*a*) to the first diffraction ring mainly as the result of obscuration, and (*b*) to a smaller extent, beyond the first ring, mainly as a result of spherical aberration and coma (D. S. GREY[27]). Condition *a* will seriously affect resolution and, therefore, the use of this system for photomicrography, while it will limit the minimum area to be used in microspectroscopic measurements to not less than that included in the first diffraction ring, roughly $2{\cdot}2\lambda/\text{N.A.}$ or 0·5 to 1μ at 2500Å. This, in fact, is approximately the limit set by the optical and geometrical characteristics of the usual biological object[17,19]. Outside the small permissible field, the rapidly increasing effects of coma with field diameter* on scattering of energy into the outer diffraction rings of the image (condition *b*) make it impossible to discover the relationship between object and image densities in small objects.

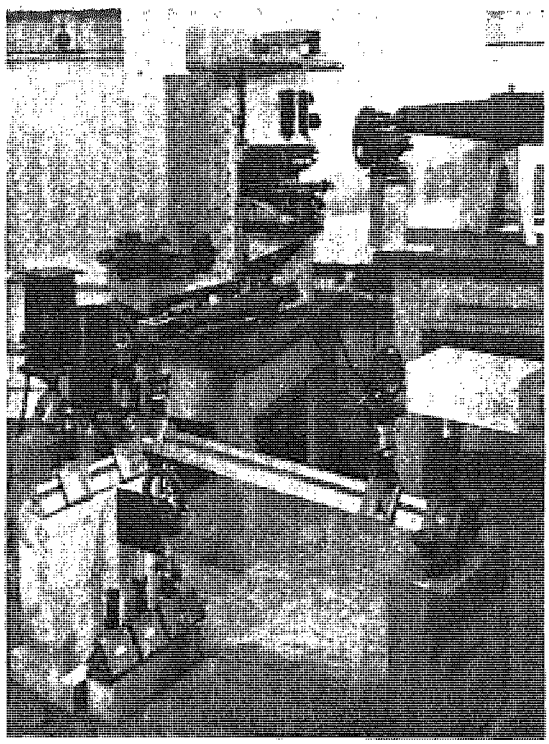

Figure 1. Burch microscope, Littrow monochromator and sources for visible and ultraviolet photomicrography; spectrograph (top right) with rotating sector in front of entrance slit is used for microspectrography

The defects of these systems are almost completely removed in aspheric reflecting objectives, of which those constructed by C. R. BURCH[23] are generally agreed to represent the highest development so far. Systems of N.A. 0·58 and 0·65, having the large concave mirror aspherized, were exhibited[30] in 1939.

* Coma causes loss of central energy mainly proportional to the square of distance from centre of field (D. S. GREY[27]).

Their N.A. may be increased by a factor 1·5 by an immersion lens component, a hemisphere with centre of curvature at the object, and the system will transmit to 1200Å if this lens is made of fluorite.

The 0·65 N.A. objective has very low central obstruction (0·14, compared with 0·45 of N.A. for the monocentric mirror-pair) and a coma-free field of 300μ diameter, the size of which is only restricted by curvature of field. This microscope, therefore, is an ideal instrument for photomicrography and quantitative microspectroscopy, to the limit of resolution of its N.A. A higher N.A. objective (0·95, increased to 1·4 by immersion) which must have both mirrors aspherized is under construction by Burch and his colleagues. The field curvature in these dry objectives is largely removed and constant alignment of the two mirror surfaces is obtained in the solid semi-reflecting (dry and immersion) objective designs of R. L. DREW[31], again having one aspherized surface (*cf* also the solid spherical mirror objective[32] of M. H. F. WILKINS and K. P. NORRIS).

It must not be forgotten that any reflecting objective used with a cover slip must be adjusted to correct the spherical aberration introduced by the latter *i.e.* by altering the mirror separation. Since this correction will vary with cover slip thickness it is important to employ a specimen cover slip of the same thickness as that on the test slide used for focusing *etc*[29,33]. A small chromatic aberration is introduced by an unimmersed cover slip and is compensated by inserting a lens element into the system or by making the small predetermined focusing adjustment by means of a calibrated fine motion[29].

An alternative to these systems is the refracting-reflecting objective, combining one or two mirrors with refracting components which may serve both as light-converging elements and to correct aberrations without using aspheric surfaces. The lenses can be of quartz, fluorite or lithium fluoride for transmission into the ultraviolet region, and the designs vary as to chromatic correction, which is not as high as in purely reflecting systems. Those objectives with a large number of lens–air interfaces will cause more light scatter, of course, but they can be made to provide long working distance, low obscuration, large field of view, and protection of the mirror surfaces, together with dimensions such as can be employed on an ordinary microscope stand. Their potential usefulness is very considerable.

One deficiency of all reflecting systems should be mentioned *i.e.* the impossibility, by the use of conventional optical filters, of separating fluorescence from absorption effects in photomicrography in some spectral regions. Thus, the fluorescence emission from an unstained self-fluorescing object, which is irradiated with ultraviolet radiation for

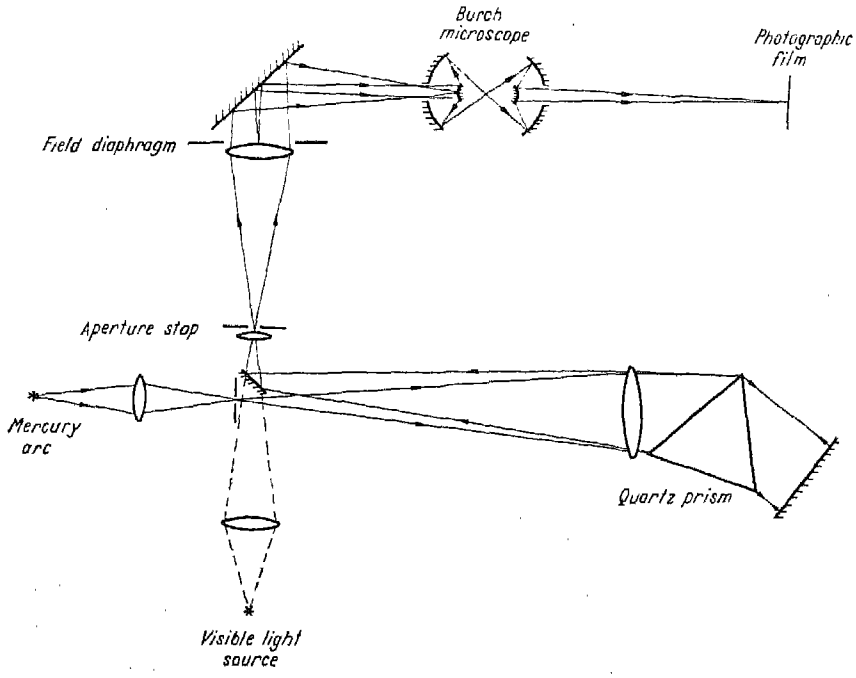

Figure 2. Diagram of optical system for photomicrography with the Burch microscope

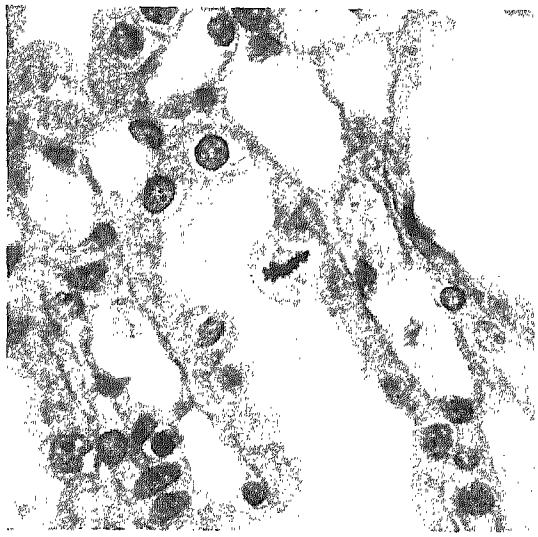

Figure 3. Visual photomicrograph of rat kidney section, stained by modified Masson's triple stain (kidney showing action of 4-styryl-2, 5, 6-triaminopyrimidine); Burch microscope, N.A.$_{obj.}$ 0·65, N.A.$_{cond.}$ 0·42; white light (×680)

the purposes of ultraviolet micrography, is brought to a focus together with the transmitted ultraviolet radiation and confuses the resulting image. It is difficult, except in particularly favourable cases, to filter the longer-wavelength fluorescence radiation without cutting out completely the ultraviolet radiation which produces it and usually is absorbed even more strongly by the filter.

SELECTED APPLICATIONS

Some examples of investigations employing a reflecting microscope will illustrate the preceding discussion. It is convenient to separate micrography from microspectroscopy as the two main fields of application.

Micrography—If a well corrected image is to be obtained with a mirror-pair reflecting objective, conditions of illumination and careful adjustment of the relative positions of the two mirrors are very important[6,28,29]. For optimum illumination a reflecting condenser is employed, although achromatic refracting condensers have been used in the visible region and an achromatic quartz–fluorite–rock salt condenser designed by Grey was used by Loofbourow[5] from 2300 to 7000Å. Accessory optics for illuminating the condenser may consist of reflecting or refracting elements, the former avoiding the necessity of refocusing with change in wavelength, and the refracting system giving the advantage of compactness. An example of the latter, employing Köhler illumination, is shown in *Figures 1* and *2* as used with a Burch microscope for visual and ultraviolet micrography. This arrangement[29] allows even illumination over the object field, *e.g.* over about 150μ diameter in the ultraviolet, using a Littrow monochromator and a medium pressure mercury arc (a line source with no appreciable continuum). Unfiltered white light from a tungsten filament lamp may be employed for visible illumination. Adjustment of a mirror-pair reflecting objective[28,29] is best performed by means of a pin-hole slide or a ring slide (consisting of a number of concentric rings intersected by a cross), the controls provided allowing (*a*) centring of the small mirror relative to the large concave one, to correct for coma, and (*b*) alteration of the mirror separation to correct for spherical aberration.

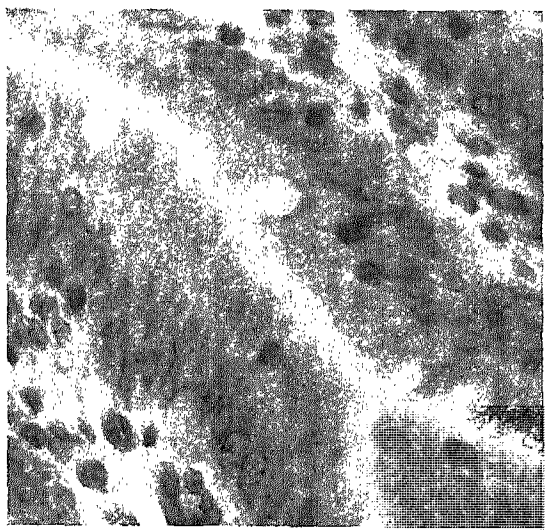

Figure 4. Ultraviolet photomicrograph of section of frozen-dried small intestine of guinea pig mounted in glycerol; Burch microscope, N.A.$_{obj.}$ 0·65, N.A.$_{cond.}$ 0·42; λ 2653Å (×680)

For photomicrography in the visible and ultraviolet regions the purely reflecting systems are commonly used without an eyepiece in order to avoid chromatic and other aberrations inherent in the latter. Thus, low magnifications are employed (*e.g.*× 110 at N.A. 0·65 and optical tube length 34·5 cm on the Burch microscope), together with fine-grain film and developers of varying contrast according to the spectral region isolated for illumination. *Figures 3* and *4* show examples of results obtained on biological sections with the 0·65 N.A. Burch objective, in the visible and ultraviolet

regions, using the illuminating system of *Figure 2*. This instrument has identical condenser and objective mirror-pairs and the system is corrected to $\lambda/10$ or better. Ultraviolet micrographs obtained with a spherical mirror objective N.A. 0·6 and with the 0·72 N.A. refracting-reflecting system of Grey have been published previously by M. H. F. WILKINS[28] and by R. C. MELLORS[34] respectively. The incorporation of visual phase contrast accessories in the Burch reflecting microscope has been described by R. J. KING[35]. Apart from their use in visual photomicrography they are necessary for searching and focusing fresh biological material prior to ultraviolet micrography. Ultraviolet phase contrast components have also been used with a spherical reflecting objective by A. H. BENNETT *et al*[36], who noted sharpening of detail in the micrograph compared with a visual phase contrast picture.

Elimination of quartz from the optical components has made possible photomicrography in the vacuum ultraviolet region[37]. For this work, oxygen must be removed from the optical path since it absorbs radiation between 2000 and 1000Å, although nitrogen is relatively transparent in this region. B. K. JOHNSON[37] has simplified the technique very considerably by enclosing the source (a high discharge spark between electrodes of tin, cadmium, aluminium or zinc), illuminating optics, microscope and the photographic plate in a chamber which is exhausted to about 10^{-2} mm Hg, flushed with purified dry nitrogen and exhausted again to a pressure sufficient for the proper functioning of the spark (about 2 mm Hg). *Figure 5*, reproduced from this work, shows the resolution and differential absorption effects obtained with this system at wavelengths down to 1438Å. For high intensity of illumination a fluorite refracting condenser was used

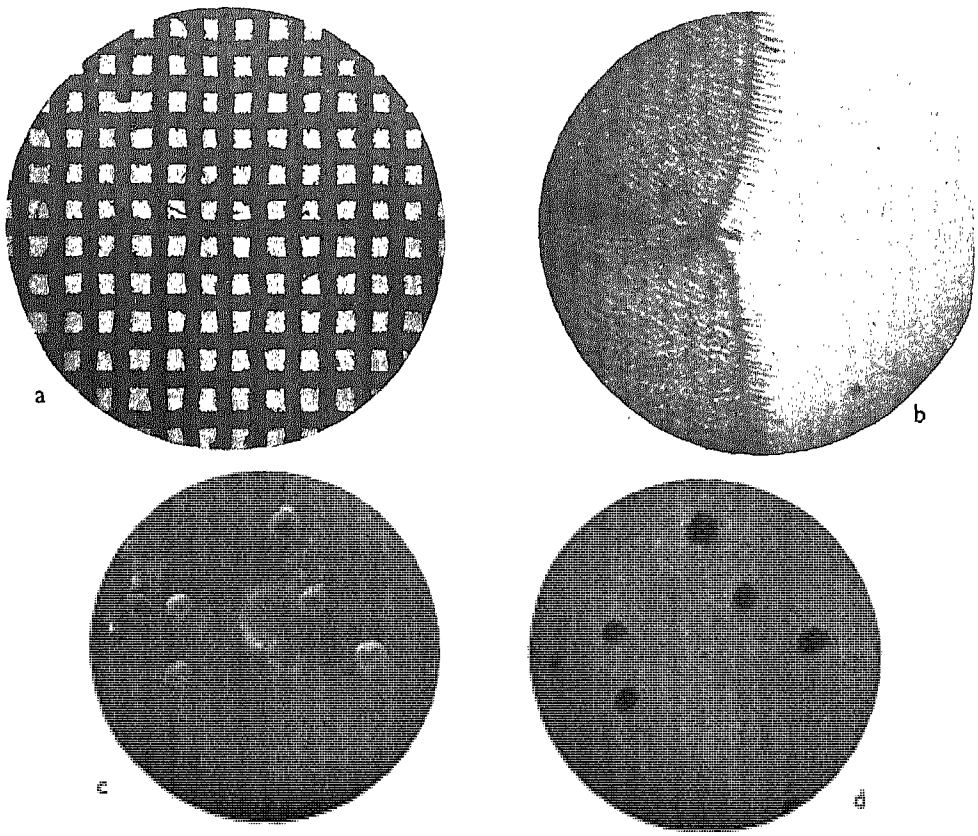

Figure 5. Vacuum ultraviolet photomicrographs using reflecting objectives (after Johnson[37]): a *copper gauze (each square of sieve=0·062 mm), N.A.$_{obj.}$ 0·25, λ 1438 Å (×35);* b *trailing edge of fly's wing, N.A.$_{obj.}$ 0·66, λ 1609 Å (×185);* c *particles of aluminium oxide* (Sira *abrasive), N.A.$_{obj.}$ 0·66, λ 4358 Å (×185);* d *as* c, *λ 1458 Å (reproduced by courtesy of the Royal Microscopical Society)*

rather than a reflecting type, owing to some loss of light in the latter through the occlusion of the central part of the illuminating beam by the convex mirror.

The necessity of delivering a minimum dose of radiation during ultraviolet micrography of living cells has led to the search for an ' image converter ' more sensitive to ultraviolet radiation than the photographic plate or fluorescent screen. Ultraviolet sensitive television camera tubes have been developed recently in the United States[38] and in Great Britain*, and used in conjunction with reflecting microscopes. Micrographs obtained with the American Vidicon tube at 4000 and 2537Å have been published for unstained fixed tissue, and these authors have also attempted to use the camera in the Brumberg–Land colour translation process to give colour television records of tissues. The British (E.M.I.) camera tube is employed in an ordinary commercial television channel. It has a useful response down to 2300Å, so that many micro-organisms, for example, have grown apparently normally with continuous irradiation for several hours at 2537, 3100 or 3650Å at radiation levels sufficient to operate the tube. The resolution of the television system is better than that required to reproduce all the detail that can be resolved at 2537Å, and the potentialities of this technique are of considerable importance†.

In the Brumberg[39] colour translation process, contrast due to selective ultraviolet absorption in the object is observed visually in colour by an ingenious method. Micrographs of the object taken at three different ultraviolet wavelengths are combined with filters in such a way that a multi-coloured projection image is viewed on a screen. Variations in hue correspond to absorption differences at the selected wavelengths and the hue at each point in the image is related to the local chemical constitution. E. H. LAND *et al*[40], using reflecting-refracting optics, have mechanized the procedure so that only a few minutes elapse between selection of the object field and the production of a colour picture, although a quantitative interpretation of such an image has not been attempted.

While the applications described so far have taken advantage of the achromatism of the reflecting microscope, two examples may be given in which long working distance has proved especially useful. K. W. KEOHANE[41] was able to use a Burch microscope for high power photomicrography (×700 at 0·65 N.A.) of hot metal surfaces, *e.g.* nickel, steel and molybdenum, by mounting the microscope horizontally. W. J. BATES and G. P. S. OCCHIALINI[42] employed the same microscope to examine nuclear emulsions at high magnification, reversing the normal procedure by inverting the plate, illuminating through the cover slip and observing through the backing plate. Thus, under oil immersion, they were able to observe multiple stars and heavy fragments not visible by the normal method of examination because they are obscured by the central body of the star. The long working distance of the microscope allows the examination in depth of emulsions so thick as to be beyond the range of ordinary objectives.

In the infra-red region the possibilities of microscopy with the Burch microscope have been explored by R. BARER[43], at wavelengths up to about 1μ. He employed a photo-electric image converter of conventional type giving an image on a fluorescent screen, as well as infra-red sensitive photographic plates, and examined stained and unstained biological material with phase contrast, polarized and unpolarized infra-red illumination. Because of reduced resolution at these wavelengths, these methods are only valuable if some other property of the specimen is important, *e.g.* relative transparency to the near infra-red compared with shorter wavelengths, such as would occur in heavily stained specimens, or infra-red birefringence as in some crystals and minerals.

Microspectroscopy—The various well known spectroscopic techniques form powerful analytical and research tools in the macroscopic field, and the reflecting microscope greatly extends their possible applications on a micro-scale. Three usual methods of arranging the component apparatus for microspectroscopy may be summarized as:

(*1*) illuminating the microscope with monochromatic radiation and focusing part of the microscope image on a radiation detector, so that a quantitative measurement of transmitted radiation is made, point-by-point at different wavelengths, as when using a photo-electric spectrophotometer in macrospectroscopy (Caspersson employed monochromat objectives in this way in the ultraviolet region)

(*2*) a portion of the microscope image, illuminated by the full radiation from the source, is focused on the slit of a spectrograph and the resulting spectrum recorded photographically; this requires an objective well achromatized and corrected for other aberrations throughout the wavelength range investigated, and has been used with reflecting objectives in the ultraviolet and visible regions

* Developed by J. D. MCGEE at E.M.I. Research Laboratories under C.V.D. Admiralty Contract.

† The author is indebted to B. ROSS ROBINSON for supplying this information.

(*3*) the full radiation from the source is incident on the specimen as in (*2*), and the radiation output from the microscope is fed into a monochromator and so to a radiation detector; macro and micro infra-red spectra are traced thus.

Optimum illumination of the microscope is still important in microspectroscopy, and the conditions to satisfy this, together with the specimen magnification necessary to fill the slit of the spectrograph and the conditions for filling its aperture with light in method (*2*), have been discussed[5] by Loofbourow. Normally much higher magnifications are used (*e.g.* ×100 to ×200 or more) than are optimal for illuminating the collimator of the usual prism spectrograph. This results in a reduction in spectral resolving power, the importance of which will depend on the nature of the investigation.

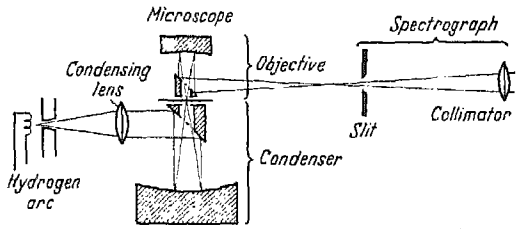

Figure 6. Diagram of simple system for micro-absorption spectroscopy of single crystals etc (after Loofbourow[5])

Quite simple, low N.A. reflecting systems may be useful for many purposes, and one is shown in *Figure 6* with the accompanying components for micro-absorption spectroscopy. Designed by T. DUNHAM, this single spherical mirror objective (N.A. 0·16) has been used to examine homogeneous objects (crystals, solid films and solutions in micro-cuvettes) in which larger areas may be selected than is usual in biological material. Loofbourow arranged this microscope as in (*2*) above, imaging the specimen on the slit of a spectrograph and taking spectrograms of the continuous ultraviolet emission spectrum of a hydrogen arc with (*a*) superimposed upon it, the absorption spectrum of the part of the object imaged on the slit, and (*b*) for comparison, a non-absorbing portion of the field, the specimen having been moved aside. By this means, on account of the achromatism of the reflecting system, the plate records in a single exposure through the specimen an infinite number of monochromatic photographs of the selected region of the object. The comparison spectrum is obtained conveniently by exposure through a rotating step- or logarithmic-sector placed in front of the slit, so that plate response to known densities is recorded. Using a microphotometer, the optical density of any portion of the image may then be found by comparison with a known density recorded at the same wavelength. The method just described has been used with objectives of higher N.A. for the micro-absorption spectroscopy of cells. R. BARER and his collaborators[44] employed a Burch microscope 0·65 N.A. in the visible and ultraviolet regions to examine human and salamander red blood cells, and these authors discuss in detail their experimental technique.

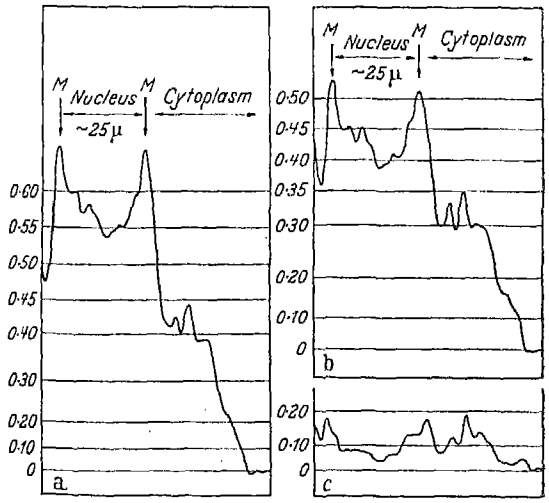

Figure 7. Microphotometer traces across spectrogram images of part of an amoeba (scales show absorption densities obtained from plate calibrations by rotating sector) at wavelengths of: a 2653Å; b 2804Å; c 3341Å

Figure 7 shows microphotometer traces* obtained similarly from the absorption images at 2653, 2804 and 3341Å of nucleus and cytoplasm of an amoeba. The unfixed cell was pressed to a thickness of about 30μ in a hole in a *Cellophane* spacer between slide and cover slip; its normal cytoplasmic streaming had been reduced after observation by visual phase contrast and ultraviolet micrography, although its morphology had not yet been affected by irradiation. The microphotometer traces were made along the length of each emission line *i.e.* in the morphological dimension. For this work the source was a medium pressure mercury arc, giving well separated sharp lines at these wavelengths, so that a single exposure (4 sec.) could be used with a relatively wide slit (0·3 mm) without danger of superimposing the absorption images due to successive wavelengths. The latter error occurs when illuminating heterogeneous objects such as biological material with a

* Unpublished work by R. J. KING, using a Burch microscope.

continuous spectral source (*e.g.* a xenon or hydrogen arc) unless a narrow slit is used. Subsequent to the exposure through the specimen, a rotating sector having density steps of 0·1 over the range 0·1 to 1·0 (a convenient range for use with biological material) was used for plate calibration, exposing through the same portion of the slit as for the specimen image. In *Figure 7* the sharp absorption peaks at *M* represent the nuclear membrane, densely absorbing at 2653 and 2804Å. The absorption of the nuclear contents is higher than that of the cytoplasm at these wavelengths also. The low absorption throughout the cell at 3341Å may be non-specific, due to light scattered at boundaries within the cell at which sharp changes of refractive index occur, although the possibility of materials being present with some specific absorption at this wavelength cannot be excluded. A continuous absorption curve of any selected area of the cell could be obtained using a hydrogen arc, for instance, as a source, by tracing longitudinally along the wavelength axis of the plate *i.e.* in the spectral dimension.

Work on liver cells using spherical reflecting systems of 0·6 N.A. has been described by H. R. CATCHPOLE and I. GERSH[25] and a similar objective was used to measure the ultraviolet absorption spectra and dichroic ratios of microcrystals and films of biological interest *e.g.* tobacco mosaic virus and calf thymus nucleoprotein[25,45]. R. C. MELLORS[6,34,46] has used refracting-reflecting and spherical reflecting systems to measure ultraviolet absorption in a variety of fixed and unfixed biological material. His work on the cells of cervical mucosa from normal and cancer patients was extended to measurements of fluorescence intensity when the cells were stained with a fluorescent dye, in attempts to devise a simple diagnostic technique.

In any such work with fresh biological cells, the effects of radiation need careful testing. They can vary considerably for cells of different types and maturity, and changes in morphology or in absorption may not appear for some time after irradiation, depending on dosage and on the wavelength used *etc.* Some investigations of these changes and of the doses tolerated by different cells have been reported[34,47,48]. Similar difficulties can occur when working with photosensitive crystals and solutions. However, such photochemical changes can give useful information if combined with the results of other experimental techniques. If sufficiently intense sources and sensitive detectors become available, photo-electric recording, using arrangement (*1*) described earlier, may be the least damaging method of determining these absorption spectra. A system for continuous recording in the visible and ultraviolet regions, using a reflecting microscope, has been developed by J. F. SCOTT and R. L. SINSHEIMER[5,49].

Quite apart from these effects, the physical and chemical complexity of biological material makes quantitative interpretation of microspectroscopic results very uncertain. In most cases, it is impossible to make the same assumptions regarding the change of spectrum with change in chemical content of the cell as may be made in macrospectroscopic work, which deals with dilute solutions under well defined optical conditions. Each cytochemical application should be considered separately. The sources of error inherent in these studies have been discussed in some detail in recent years[25,27,47,50]. It is clear that quite large changes in, for example, the ultraviolet absorption of a cell structure may indicate only trends of chemical change and should be interpreted with caution. Nevertheless, this information may not be obtainable in any other way and if combined with other cytochemical data may have considerable importance.

The first infra-red microspectroscopic studies were made by R. BARER, A. COLE and H. W. THOMPSON[51], employing a Burch microscope to examine, for example, single crystals and oriented fibres (including nerve and muscle). In this region of the spectrum the use of the reflecting microscope is rather different from that at shorter wavelengths. Introduction of reflecting optics made possible long-wavelength infra-red microspectroscopy but, mainly on account of the wavelengths used, the microscope functions as a micro-illuminator with which to observe infra-red absorption, rather than to resolve detail in the object. Aberrations which are important in the ultraviolet region are relatively unimportant at these wavelengths, where the dimensions of the diffraction pattern are greatly increased. A spherical reflecting system is quite adequate up to 0·65 N.A. and it may be combined with a plano-convex lens of silver chloride or thallium bromoiodide to give numerical apertures as large as are allowed by the refractive indices of the liquids used.

The apparatus for infra-red microspectroscopy is usually arranged as in system (*3*) described earlier, but the monochromator can be placed before the microscope[52] if it is undesirable to subject the specimen to the full radiation from the source throughout the scanning of the spectrum. There has been detailed discussion of optimum conditions of illumination, matching of spectrometer and micro-illuminator components, magnification, and minimum size of specimen *etc* by E. R. BLOUT, G. R. BIRD and D. S. GREY[53], amongst others.

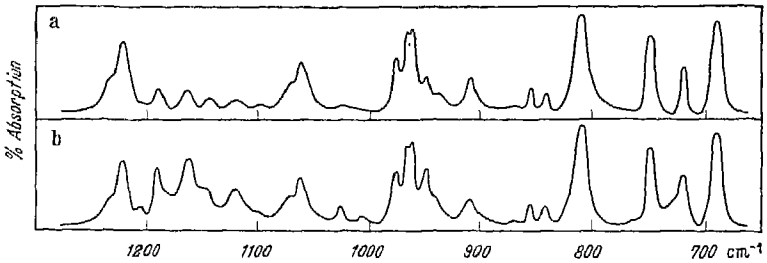

Figure 8. Infra-red spectra obtained from : a *a single crystal of 4-dimethylaminostilbene, using a microspectrometer ;* b *the same compound as a* Nujol *mull, by normal macro-operation*

These workers linked a commercially available spectrometer and microscope, as did R. C. GORE[54], while R. B. D. FRASER[25] used specially designed components together with a commercial monochromator to give an apparatus of high spectral resolution. It has to be remembered that it is the energy available rather than the resolving power of the microscope which sets a lower limit to the cross-sectional area of the specimen to be examined. Furthermore, thickness of specimen may have to be chosen to give an optimum absorption value. Size of specimen and resolving power of the microspectrometer are discussed, therefore, in terms of the smallest volume which can be examined with satisfactory signal to noise ratio rather than in terms of the smallest area. Further increase in this ratio may be gained by decreasing the area of the detector or increasing the running time to scan the spectrum.

The wavelength range of interest in the infra-red spectroscopy of organic molecules is large (2 to 30 μ), so that if it is required to examine such material from, say, 2 to 16 μ the minimum linear dimensions of the object permitted by diffraction theory will vary about eightfold over this region, according to equation 1. Further, the resolving power of a microscope of 0·6 N.A. is equal to the wavelength used, so that at this N.A. observations around wavelength 10 μ on biological material would require an area 10 μ in diameter, a size covering nucleus and cytoplasm of a single cell in a number of cell types. In practice, the minimum areas measured so far have been many times larger than this. If Caspersson's calculations[17] at shorter wavelengths also apply in the infra-red region, then a size of 4λ would be required for a quantitative absorption measurement, so that the resulting spectrum frequently would be an average over the contents of a number of cells.

Apart from this lack of morphological resolution, there are other limitations to infra-red investigations of biological cells such as, in fresh cells, the need to examine the possible effects of the radiation employed, and the presence of water in the cell and suspending medium, giving strong interfering absorption bands around 3 and 6 μ. The latter effect could be eliminated by comparison with results in deuterated water, but this may not often be possible in biological work. Further, there is at present limited understanding of relationships between changes in infra-red spectra and chemical structure in many of the macromolecules which occur in biological material (proteins, polynucleotides, lipids *etc*). Nevertheless, samples of blood, or bundles of cells such as nerve or muscle fibres[51,55] and tissue sections[27], have been examined, and it has been noted that some plant tissues of specialized type give characteristic spectra *e.g.* leaf cuticle and the immediately underlying epidermis show distinct spectral differences[25].

Applications to single crystals and other microquantities of material are much more promising, and—where the specimen is oriented—dichroism studies may be carried out, using silver choride sheet or selenium film polarizers. The aim in this work is to retain a spectral resolution approximating to that used in macrospectroscopy, so that sharp absorption bands are not obscured (a more frequently important requirement in infra-red spectroscopy than at lower wavelengths) and, if necessary, comparison may be made between the microsample and the same or similar material as solution or paste (mull) examined on the macro-scale.

Figure 8 shows the spectrum of a crystal* of 4-dimethylaminostilbene, one of a series of carcinogenic and tumour-inhibitory derivatives of stilbene. The spectrum is compared with that of a mull of the material in liquid paraffin, and shows the resolution which may be given by a simple microspectrometric arrangement if useful magnification is not exceeded. The apparatus employed in this work linked a commercially available spectrometer and microscope *i.e.* a Perkin Elmer single beam spectrometer and a Beck spherical reflecting microscope of 0·65 N.A. and 35 per cent central obstruction. The latter was arranged between source and monochromator with accessory mirrors to give a magnification of about 7 times between the specimen and

* Part of an investigation, in progress, by S. F. D. ORR, who also designed the instrumental arrangement.

the entrance slit of the spectrometer. This is the useful magnification for a spectrometer of this type (equivalent N.A. about 0·1), thus giving full illumination of the collimator and optimum spectral resolution. Objects down to 0·5 mm long may be used under these conditions.

CONCLUSION

In 1943, C. R. BURCH[30] stated that only experience with different microscopic objects and types of illumination would show whether the labour of aspherizing a mirror-pair objective was worth while. He pointed out that other methods could be used to maintain optical tolerances in reflecting systems, and time would show whether they could be applied more simply. Since then, the achromatic refracting-reflecting microscope has been produced and found to be a useful alternative, for many but not all purposes, to the well corrected aspheric reflecting systems; the spherical reflecting objective has proved valuable for work with long-wavelength illumination and its useful applications at shorter wavelengths have been defined. Sufficient experience is now available to underline the factors which need consideration before choosing a reflecting microscope for a particular application. In micrography, a vast field of research awaits exploration in the examination of fresh biological material by means of ultraviolet radiation. In microspectroscopy, if the limitations of the technique in various spectral regions are understood, a sensitive tool is available which, in combination with other techniques, can give information at present not obtainable by any other method. As these potentialities are recognized, it is to be expected that the reflecting microscope, particularly of high numerical aperture, will be increasingly in demand.

The author is indebted to Professor B. K. Johnson and to Dr T. Dunham, Jr, for permission to reproduce Figures 5 and 6, and to Mr R. J. King and Mr S. F. D. Orr for providing Figures 7 and 8.

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FLUORESCENCE MICROSCOPY

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An account is given of the mechanism of fluorescence microscopy, supplemented by the author's experience and measurements. Selected applications of the technique are described—fluorescence microscopy enables substances to be detected which are not visible under the ordinary microscope.

FLUORESCENCE MICROSCOPY has until recently been the monopoly of a few specialists. The pioneer work of M. HAITINGER, P. ELLINGER and A. HIRT[1–5], A. GRABNER and others is relatively unknown: it has been only partly recorded, and then in rather obscure journals. Few microscopists are also physicists and *vice versa*, but the establishment of a chair of Technical Optics in the University of London should do much to develop and popularize the scientific and technical applications of specialized microscopy. The literature on fluorescence microscopy is widely scattered in diverse scientific journals, and is not always reliable. The microscopist—frequently a botanist, biochemist or a medical man—rarely has access to the physical instruments essential for checking characteristics of ultraviolet lamps, filters, lenses *etc*, and so is dependent on commercially constructed apparatus. While some modern commercial units are of excellent design, they are expensive and their construction may not be suitable for all the applications of fluorescence microscopy. The time is rapidly approaching, however, when this form of microscopy must be regarded as a necessity rather than a rarely attainable luxury.

In general, the main advantage of fluorescence microscopy over ordinary microscopy derives from the fact that it enables fluorescent materials in animal and plant structures to be located. Further, nearly all the structure of many specimens of plants, animals *etc* fluoresces to some extent—although the fluorescent substances may be present in minute amounts and their nature unknown—and certain fine structure may be therefore revealed which cannot be observed by normal microscopy. For example, certain structures can be seen in a cross-section of a wheat grain, especially when treated with alkaline potassium ferricyanide solution which converts aneurine into strongly fluorescent thiochrome. Not only is the structure containing aneurine shown in excellent relief (mostly the scutellum) but other structures are also better defined. If care is taken to avoid adventitious fluorescence due to dust, mounting media, microscope slides, lenses *etc*, carefully filtered ultraviolet incident light can be made to give a practically black background. Only fluorescent structure then affords visible light and the eye is not distracted by an overwhelming amount of non-fluorescent visible light which contributes nothing to the definition of the structure—it frequently detracts from adequate definition even in normal microscopy. It is thus possible to observe extremely small differences in intensity aided by colour, and to ensure the maximum contrast. Contrast perception may be greatly augmented by adapting the eye to the dark before observing the object. Fluorescence microscopy is therefore best carried out in a dark room, the dark adaptation of the eyes occupying about half an hour. This procedure may only be justified on rare occasions, but can lead to rewarding results.

The retina is most sensitive to colour vision in the yellow-green region of the spectrum, being a maximum at a wavelength of 555 mμ and decreasing rapidly with increasing or decreasing wavelengths. The energy required for colour vision to be operative in the region below 440 mμ and above 660 mμ is very much greater than in the region 530 to 580 mμ[6]. The tendency is to report yellow or green fluorescence but to overlook the presence of objects which only afford feeble blue or red fluorescence. Every effort should therefore be made to increase the intensity of the ultraviolet light and to exclude extraneous visible and fluorescent light.

APPARATUS

Microscope—Most modern microscopes are suitable for fluorescence work but it is preferable that neither the objectives nor the lining of the microscope barrel fluoresce in ultraviolet light. Ultraviolet

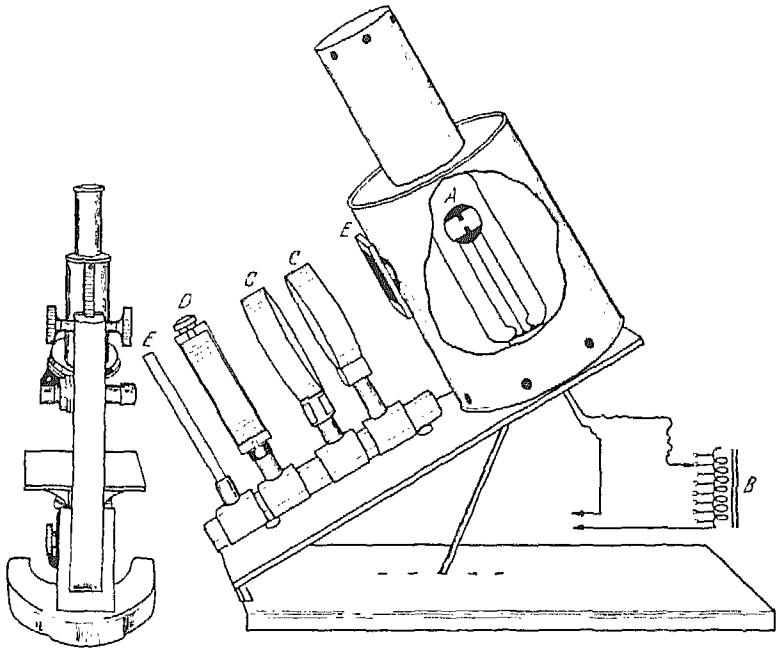


Figure 1. Microscope: A compact source high pressure mercury vapour lamp (250 w., ME/D type); B choke; C quartz lens; D liquid filter cell; E glass filter

illumination may be obtained by sub-stage transmitted light or by incident light from above. When sub-stage illumination is used, the condenser should be of quartz or glass which will freely transmit ultraviolet light of the desired wavelength.

J. SMILES[7] has described an efficient dark-ground ultraviolet illuminator which incorporates a combination of a quartz prism and aluminized concave mirrors. Several alternatives are available for the use of incident ultraviolet light directed from above the object. The simplest is the Lieberkühn reflector with an aluminized surface. Such a contrivance, or any method which relies on a sub-stage illumination, cannot be used if a large section or object that does not transmit ultraviolet light is to be examined. In these circumstances the source of ultraviolet radiation must be above the object. When a long working distance objective can be tolerated, the ultraviolet light may be focused directly on the object (*Figure 1*). By fixing the Dyson[8] long-distance device to the objective the working distance is appreciably increased, but some loss of visible light results. Alternatively, a small aluminized or specially treated mirror can be placed at a convenient angle below the objective. Such a system requires a compact source of ultraviolet light and accurate focusing by well designed quartz lenses.

When higher magnification is required a more elaborate system may be necessary. The least complicated of these is the vertical side arm illuminator, in which the ultraviolet light is injected through an aperture in the fitting, and is deflected through the objective by a quartz or glass plate or by a prism (*Figure 2*). The efficiency of the plate system has been greatly increased by J. R. BENFORD[9] who deposited magnesium fluoride on the upper surface and titanium oxide on the lower surface. It is claimed that, by careful control of the thickness of these films, an overall efficiency of about 23 per cent is achieved, whereas that of the plain quartz or glass plate is only about 5 per cent. Part of the plate may be half-silvered by deposition of aluminium *in vacuo*. This method can lead to considerable distortion of the image, and various aluminized segments or arcs of the plate have been advocated

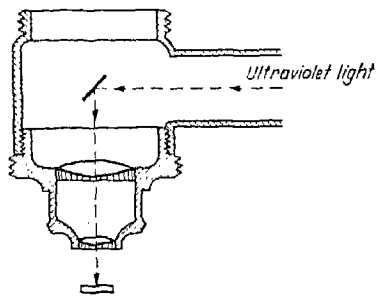


Figure 2. Vertical illuminator with aluminized mirror

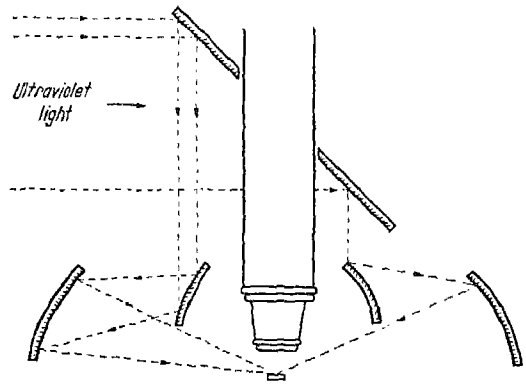


Figure 3. Chapman and Alldridge illuminator

instead of the plain half-silvering, but the method is not satisfactory for critical work. In this system the objective acts as a condenser, so that the correct cone of ultraviolet light must strike the plate and the objective must transmit ultraviolet light freely. It is convenient to use objectives with shorter mountings than normal, to accommodate the side arm illuminator, and in any case the microscope tube length should be adjusted to compensate for any departure from the normal.

The most satisfactory side arm illuminator is a modification of the Chapman and Alldridge illuminator[10] with aluminized surfaces (*Figure 3*).

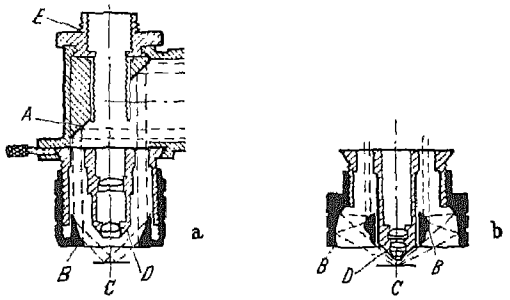


Figure 4. Beck-Chapman modification of the Chapman and Alldridge illuminator: a *normal system;* b *with additional mirrors for use at higher powers* (A *plane aluminized mirror,* B *curved aluminized mirror,* C *object,* D *special objective with narrow barrel,* E *universal thread to fit into body of standard microscope*)

The Beck modification is illustrated in *Figure 4*. Aluminized surfaces, whether on plain or concave mirrors, are prepared by first depositing a film of chromium on to the optically ground glass in a high vacuum, to ensure good cohesion and hardness. This is followed by a deposit of aluminium which reflects light extremely well, even the fairly short ultraviolet wavelengths. It has not been found practicable to construct this type of illuminator with powers higher than 4 mm owing to the increasing difficulty of adjusting visual and ultraviolet focusing in the higher powers, which must be carried out independently. It is of great assistance in focusing with any system to place some extremely small crystals of uranium nitrate on a microscope slide of the same thickness as the type on which the object is to be examined. Focusing of both ultraviolet and visible light is thereby greatly facilitated. The cone of ultraviolet light can be traced and adjusted by the aid of a sheet of uranium glass, or by a piece of filter paper moistened with acidified quinine sulphate solution.

The compact source high pressure mercury vapour lamp gives its greatest ultraviolet intensity near the pole pieces of the arc, as shown by *Figure 5*. When using this source with any of the modifications of the Chapman and Alldridge illuminator, it is therefore an advantage to arrange the microscope in a horizontal position so that the most intense ultraviolet areas strike the plane mirror and not the barrel of the objective.

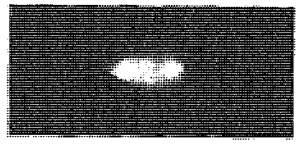


Figure 5. Ultraviolet output of compact source high pressure mercury vapour lamp showing greatest intensity nearest the two electrodes

The reflecting objective offers possibilities of overcoming some of the inefficiencies and difficulties of the side arm illuminator but has not yet come into use for the purpose. B. K. JOHNSON[11] has described a compound reflecting microscope which could be

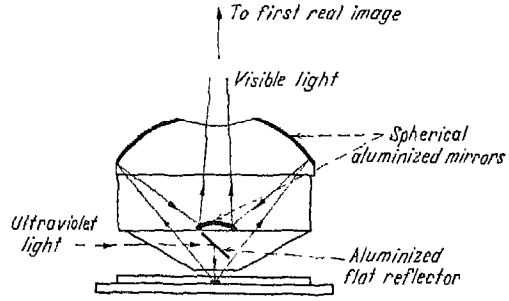


Figure 6. Reflecting objective: suggested method for use in fluorescence microscopy

readily adapted for fluorescence microscopy, and it is hoped that this will shortly be available commercially. The reflecting objectives developed[12] by the Biophysics Unit of the Medical Research Council offer distinct possibilities (*Figure 6*). These have a small dead space below the lower reflector and it should be possible to insert an aluminized plain mirror in a suitable position to reflect ultraviolet light through 45°, on to the object. This would cause complications in the Beck model (*Figure 7*) as the objective is fitted with an adjustable metal collar for ultraviolet focusing and the incorporation of a mirror would need careful designing. In recent models the aluminized reflecting surfaces are protected by being enclosed in the material of the

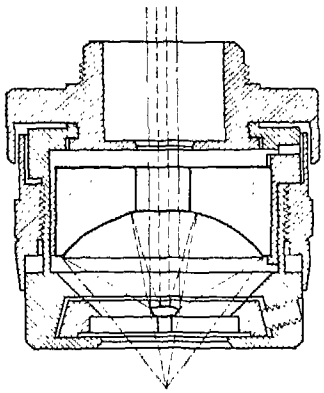


Figure 7. Beck reflecting objective

objective (quartz) and there is an increase in the numerical aperture, without increasing the central obstruction of numerical aperture. Whatever system of illumination is used, every effort should be made to minimize losses both in ultraviolet light and in the fluorescent visible light. F. E. J. OCKENDEN[13] has recently called attention to the losses due to reflection at surfaces. The loss at each surface is usually not less than 4 per cent except for ' bloomed ' surfaces, and may be considerably more for steeply curved lenses or oblique angles of incidence. The overall transmission of a 2 mm uncoated objective may be only 35 per cent, and for 4 to 8 mm objectives about 38 per cent. The cover-glass type of side arm illuminator has only about 6 per cent efficiency. To these losses must be added those inevitable in any light-filtering and condensing system. Each stage must be carefully designed, *i.e.* light source, condensing, filtering, magnification, otherwise the losses can be quite prohibitive.

Illumination—An efficient system necessitates an intense and compact source of ultraviolet light. Formerly this was provided by electric arcs such as those of cadmium, carbon, or iron-cored carbon. The modern compact source high pressure mercury vapour lamp is much simpler, providing a long trouble-free life[14]. These lamps, known commercially as the ME type, are available in a range of powers, but for most purposes the 250 w type is satisfactory. This is air cooled and partly enclosed by a metal box which acts as a shield in case the quartz envelope bursts under the heat and pressure of the enclosed gas. The commercially available higher powers are corrected for colour by cadmium in addition to the inert gas, and may lead to greater difficulties in the light filter system. The relative output of a 250 w ME type compact source high pressure mercury vapour lamp recorded by a quartz spectrograph is illustrated in *Figure 8*. It should be borne in mind that the photographic plate is not equally sensitive to all wavelengths, but the plate illustrated gives a fair representation of the lamp's output. Measurements of energy at various wavelengths, relative to the maximum output at 3655Å taken as 100, are: 50·4 at 3130Å, 19·5 at 3022Å, 12·5 at 2652Å, 9·8 at 2967Å, and 6·7 at 3342Å, with smaller outputs at some other wavelengths. The ultraviolet continuum including reversal wings at 2537Å is reported[15] to have a relative energy of 259.

The greatest amount of ultraviolet energy is therefore obtained by using the broadest band in the ultraviolet filter system and, when some visible light can be tolerated, a single ultraviolet filter of Chance OX7 (3 mm) glass unaccompanied by liquid filters is satisfactory. The compact source lamps should be operated in a vertical position, otherwise radiation reaching the quartz envelope when the lamp is used at a large angle from the vertical soon causes deterioration and destruction of the envelope. For limited periods however it is safe to use them at an angle, preferably not exceeding 15°, although precautions should be taken in case the envelope

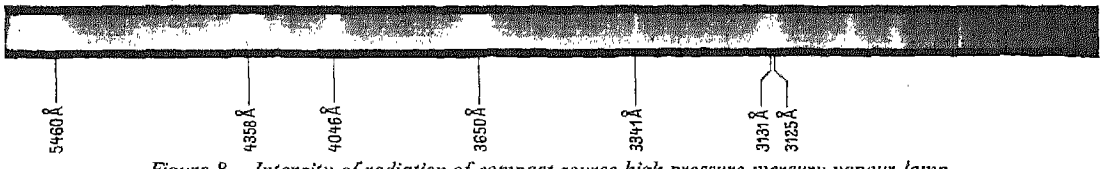


Figure 8. Intensity of radiation of compact source high pressure mercury vapour lamp (ME box type, 250 w)

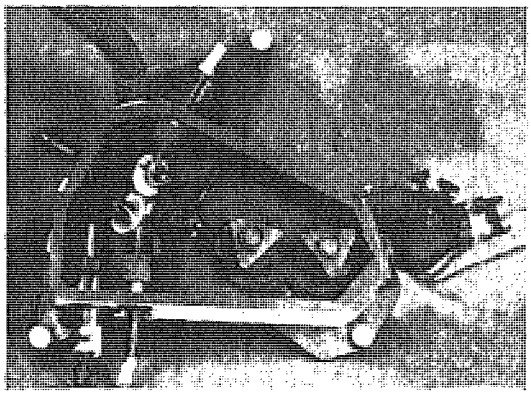


Figure 9. Combined ultraviolet unit (cover removed) consisting of an arc unit with revolving cadmium electrodes, a monochromator, a low pressure mercury resonance lamp (90 per cent of the output is confined to the frequency of 2537 Å) and a high pressure compact source mercury vapour lamp (by courtesy of Cooke, Troughton and Simms Ltd)

bursts. The ultraviolet band at 365 mμ is satisfactory for most purposes, but a cadmium arc followed by a monochromator may be necessary when substances which fluoresce at considerably lower wavelengths are to be examined. The efficiency of the mercury vapour lamps is not sufficiently high at the shorter wavelengths to compensate for the losses in the filter system which must be used to eliminate light of visible and higher ultraviolet wavelengths. Suitable cadmium arc apparatus is available commercially. When examining an object by the lower ultraviolet wavelengths a monochromator is almost essential (*Figure 9*).

Filters—A broad band of ultraviolet radiation is satisfactory for many purposes, so that it is merely necessary to filter out all visible light. This may be done either by a combination of glass and liquid filters, or by the much more expensive monochromator. The combination of glass and liquid filters can be readily constructed in any well equipped workshop, but the construction of a monochromator, which necessitates the use of quartz prisms and aluminized mirrors, is best left to commercial production. The construction of filters for critical work is difficult, since even the best commercial ultraviolet filters pass a considerable amount of visible violet, blue and red light. It is impossible to eliminate all visible light without seriously diminishing the intensity of the ultraviolet component. M. KASHA[16] has described a series of glass and liquid filters for isolating various wavebands in the ultraviolet region, but the amount of transmitted ultraviolet light is reduced to about 30 per cent of the light reaching the filters. Two of the substances he employs in the liquid filters do not appear to be marketed, but can be prepared fairly easily in a well equipped organic chemical laboratory. The amount of visible light passed by a system of filters can be approximately judged by closely examining a 100 w tungsten lamp, but it should be borne in mind that the output of violet light by such a lamp is much less than from mercury vapour lamps. The red radiations are readily removed by a 5 cm path of a 10 per cent solution of copper sulphate which transmits 73 per cent at 365 mμ, 2 per cent at 600 mμ and nil at wavelengths exceeding 640 mμ. These figures do not include the losses due to the glass or quartz ends of the cell. Removal of the last traces of violet light is much more difficult, unless considerable reduction of the 365 mμ waveband can be tolerated. As will be seen from *Table I* the Wratten 18A transmits 34·5 per cent at 365 mμ and nil at wavelengths between 400 and 600 mμ, and somewhat similar results can be obtained by two Ilford 828 (3 mm) or two Chance OX1 (3 mm) filters in series. Kasha's filter A is no better than this, and is much more complicated[16].

*Table I. Percentage Transmissions of Glass Filters**

Wavelength mμ	*Chance OX1 3·0 mm*	*Chance OX7 2·5 mm*	*Wratten 18A 4·3 mm*	*Wratten 23B10 2 mm*
254	*nil*	40	0·35	45
365	64·4	82	34·5	90·7
400		17·6	0·73	
420	0·8	0·5	0·1	
600	*nil*	*nil*	*nil*	
700	4·6	47·3	1·0	44
750	16·2			

* The thickness is only nominal *e.g.* Chance OX7 varied from 2·46 mm to 2·50 mm in different parts. It is understood that Ilford 828 has the same characteristics as Chance OX1. A 'Woods' filter had nearly the same characteristics as Wratten 18A, but specimens vary considerably. Measurements were made by the Hilger Uvispek spectrophotometer

In the author's experience, except for the most exacting work, the copper sulphate filter followed by (or with) a 3 mm Chance OX1 or Ilford 828 is a satisfactory combination. Fortunately most observers have poor colour vision in the far violet region of the spectrum, but some individuals have colour vision below a wavelength of 390 mμ and may be under the impression that they are observing blue fluorescence, whereas they are merely seeing reflected violet light which is practically invisible to the normal person. Even when the best available filters are employed, some visible light may be generated through the presence of slightly fluorescent dust on parts of the

equipment. When using sub-stage ultraviolet illumination the amount of ultraviolet light of wavelength 365 mμ reaching the eye through the microscope may be sufficient to make the lens of the eye fluoresce, giving the sensation of a pale blue background. If an ultraviolet filter is incorporated in the microscope, this will be prevented. While manufacturers do their utmost to standardize their filters, it should be remembered that the preparation of nickel-containing glasses is a subject as yet little understood, and strange results are obtained from some melts. Wherever possible, transmissions at various wavelengths should be measured before a filter is taken into use.

In devising a filter system, losses should be carefully checked, the number of glass–air and glass–liquid surfaces being reduced as far as possible.

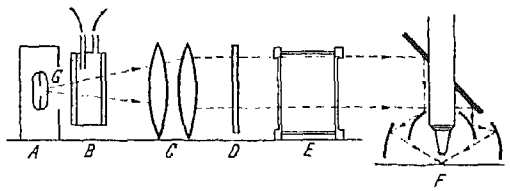


Figure 10. Filter system : A compact source high pressure mercury vapour lamp (ME box type, 250 w); B water cell heat filter (for λ band 365 mμ); C quartz lenses; D glass filter; E liquid filter (5 cm path); F Chapman and Alldridge type side arm vertical illuminator; G glass window in lamp housing replaced by quartz window

The total losses in the filter system used by the author and illustrated in *Figure 10* are high, only approximately one third of the energy which reaches the first filter emerging from the last. (Cell E must have quartz end disks for use with liquid filters to pass lower ultraviolet wavelengths; copper sulphate solution is used for the 365 mμ band and a solution of nickel and cobalt sulphates for the 310 mμ band—see *Tables II* and *III*). It is doubtful whether improved glass filters can be marketed at present, but some improvement may be possible in experimental trials. A filter 6 mm thick of an experimental melt made by H. MOORE, of the Department of Glass Technology, Sheffield, had the following excellent transmission characteristics: 44 per cent at 365 mμ, 1·9 per cent at 391 mμ, 0·3 per cent at 405 mμ and 0·2 per cent at 436 mμ. This was used to remove all visible violet light and contained calcium, boron and aluminium oxides, with a proportion of nickel.

When the apparatus has to be operated for long periods, *e.g.* for colour microphotography, the heat generated by the lamp may be troublesome and should be removed as shown in *Figure 10*. The heat removing cell has two ends of Chance ON20 or ON21 glass each 1·5 mm thick, with running cold water. The use of a single layer 3 mm thick without water frequently results in shattering the filter and, even when this does not take place, the filter becomes extremely hot and re-radiates the heat. Without adequate heat removal at this stage the copper sulphate solution in the filter combination becomes hot and may even boil. L. A. JONES[17] has published reliable figures for some filters and the transmission of an acidified copper sulphate solution is given in *Table II*.

Staining techniques—The methods used for fluorescent staining are similar to the normal techniques employed by botanists and bacteriologists, and are described by S. STRUGGER and others in *Beiträge zur Fluoreszenzmikroskopie*[18]. The uses of forty fluorescent stains are dealt with in *Fluoreszenzmikroskopie mit Fluorochromen*[19]. The colour of the fluorescence is somewhat dependent on

*Table II. Percentage Transmissions and Optical Densities of 1 cm Column of a Solution of Copper Sulphate in 1 per cent v/v Sulphuric Acid (2 per cent w/v $CuSO_4$, $5H_2O$)**

Wavelength mμ	*Optical density*	*Transmission* %
270	> 3	*nil*
280	1·225	6·0
290	0·530	29·7
300	0·222	60·2
310	0·094	80·6
330	0·030	93·3
350	0·018	95·7
365	0·006	98·0
380	0·003	99·2
400	0·0023	99·5
450	0·0011	99·7
500	0·0026	99·4
550	0·0155	96·5
600	0·068	85·5
650	0·224	59·7
700	0·527	29·7
750	0·817	15·2

* The percentage transmissions of suitable filters of acidified solutions of copper sulphate may be calculated from the figures given in the table as follows: If D_2 is the optical density required, D_1 the optical density given in the table, C half the concentration of copper sulphate in the table, and l the optical path (cm), then

$$D_2 = D_1 \times C \times l$$

This does not include losses in transmission due to surface reflection, which are approximately 4 per cent for each air–glass or glass–liquid surface (this also applies to silica) *e.g.* the optical density of a 2 per cent solution of optical path 5 cm is 0·03 at 365 mμ, 0·34 at 600 mμ, 1·12 at 650 mμ, and 2·63 at 700 mμ. Optical densities may be converted into percentage transmissions as follows:

$$D = -\log_{10}(\%\text{Transmission}/100)$$

Therefore the percentage transmissions of the above filter are 93·3 at 365 mμ, 7·6 at 650 mμ and practically nil at 700 mμ. Allowing for 4 per cent loss at each surface, without further loss for the quartz material, the overall transmissions become 79 per cent at 365 mμ and 6·4 per cent at 650 mμ. This filter in combination with the filters in *Table I* transmits ultraviolet radiation of wavelength 365 mμ adequately, while suppressing visible red light. This table shows that such a filter will seriously diminish the transmission at wavelengths below 310 mμ. Where it is essential to eliminate all visible red light, the concentration of copper sulphate should be increased to 10 per cent

Table III. Percentage Transmissions and Optical Densities of 1 cm Column of an Aqueous Solution Containing 24 per cent w/v Nickel Sulphate $NiSO_4,6H_2O$ *and 4·5 per cent Cobalt Sulphate* $CoSO_4,7H_2O$*

Wavelength mμ	*Optical density*	*Transmission* %
220	0·420	38·0
230	0·081	83·1
240	0·038	91·7
260	0·033	92·7
280	0·025	94·5
300	0·020	95·6
320	0·043	90·9
330	0·107	78·2
340	0·237	58·0
360	0·876	13·3
540	0·609	24·5
580	0·426	37·2
600	0·69	20·5
650	2·0	1
900	0·301	50·0

* This filter is unsuitable for a waveband with maximum at 365 mμ, but is suitable for a waveband from 230 mμ to 340 mμ. The filter must have quartz ends. The heat removing filter must be removed as the Chance ON20 or ON21 will not transmit the lower ultraviolet wavelengths. A 2 cm liquid filter path in combination with Chance OX7 (2·5 mm) or Wratten 23B10 (2 mm) is satisfactory. As the combination of filters does not remove heat, a sheet of asbestos card should be inserted before the quartz lenses and only removed for the short time during which objects are to be examined

conditions and may change appreciably with changing *p*H. The most useful stains are coriophosphine, acridine orange, berberine sulphate, rhodamin B and auramin (*Figure 11*).

When the fluorescent dye stains the object bright yellow or green, elaborate ultraviolet filtering is unnecessary. A Chance OX7 filter in conjunction with the 250 watt compact source high pressure mercury vapour lamp is satisfactory. Unwanted colours are suppressed by inserting a yellow or green filter into the microscope. Small bright yellow or green objects are therefore readily seen, as they are sharply defined against a practically black background, and this enables an 8 mm or even a 16 mm objective to be used in examining small objects such as bacteria. Certain organisms can thus be observed with far less fatigue than when normal stains are used with the usual 2 mm oil immersion objective, as the 8 and 16 mm objectives increase the field covered about sixteen times and sixty four times respectively. The time occupied in searching for bacteria, *e.g.* tubercle in sputum is greatly reduced, and eye strain largely eliminated. The mounting medium for specimens must be non-fluorescent *e.g.* glycerol. Commercial glycerine jelly frequently contains a fluorescent preservative.

APPLICATIONS

The earliest applications of fluorescence microscopy were in the field of biology, and P. ELLINGER[20] has provided a comprehensive review of the advances up to 1940. Some further applications have been described by F. BRÄUTIGAM and A. GRABNER[18], but more recently the technique has invaded so many fields of science that it is extremely difficult to make an adequate summary. Of the applications to biology, perhaps the most spectacular are in the field of what Ellinger describes as 'intravital microscopy'. By his method, the location of porphyrins, riboflavin, cells containing vitamin A and aneurin in animal and plant structure is made comparatively easy. Aneurine requires conversion into thiochrome, as it is otherwise invisible. Strugger has described in detail the technique for employing this type of microscopy in bacteriology, and H. Haberlandt and A. Köhler for its use in mineralogy and petrology[18].

C. A. THOROLD[21] was able to trace the movements of the sporangia of *Phytophthora palmivora* which infect cacao pods. His technique consists in spraying the infected pods with a solution of primuline, and then mounting disks of *Cellophane* covered with petroleum jelly along the surface of the pods. The 'take-off' of the sporangia is readily noted, as the sporangia caught by the disks are identifiable by their greenish yellow fluorescence. A modification of this process has been used in the United States for tracing air motion. The method, due to W. A. PERKINS[22] and F. R. HOLDEN and his colleagues[23], consists in releasing fluorescent particles dispersed from chimney stacks or effluents from industrial plant, and tracing the movements of these effluents by collection of the particles. These particles, consisting of a special zinc cadmium sulphide phosphor, are commonly 1 to 5μ in diameter, so as to remain airborne for long periods. At selected sites the particles are collected on *Millipore* filters which hold the particles on the surface with little penetration and which, having a dark blue background, provide excellent contrast for microscopic viewing. The filters are examined under near ultraviolet light using a 16 mm objective and a 10× eyepiece. It is claimed that the fluorescent particles can be measured in concentrations as low as 1 particle in 10 ft^3 of air, corresponding to a mass concentration of the order of one part in 10^{13}. J. KING and R. E. WESTON[24] have described the detection of aneurine hydrochloride (vitamin B_1), a solution of which was sprayed on to wheat flour. The flour is spread in a thin layer on to a sheet of glass which has been flooded with a diluted solution of alkaline potassium ferricyanide. The excess flour is removed and the lower surface examined under ultraviolet light by a low power binocular. Aneurine is thus converted into thiochrome which can be

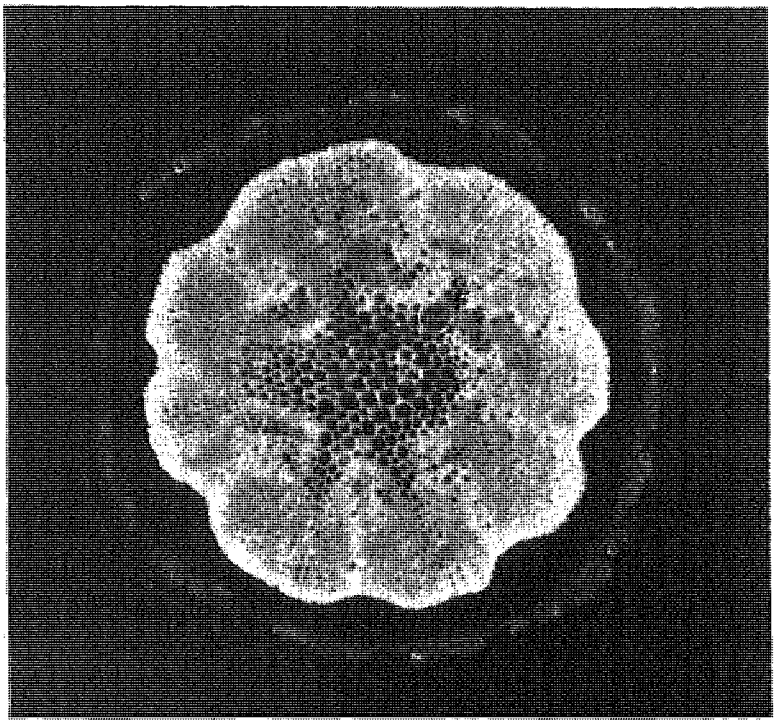
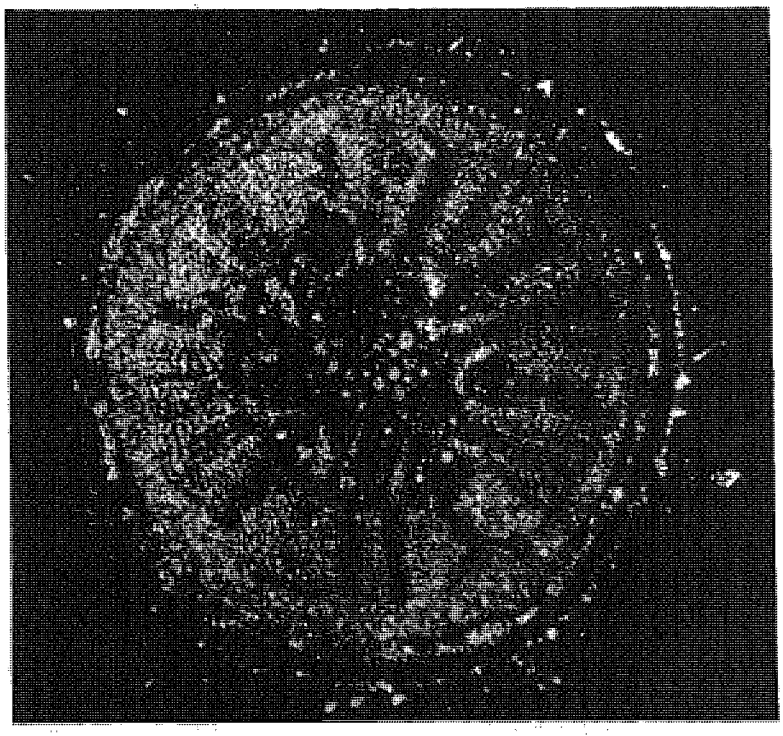


Figure 11. Photomicrographs of a cross-section of stem of Berberis Darwini *cut by hand, 40 mμ thick, mounted in glycerol but otherwise untreated*

◀ a *fluorescence microscopy*



▶ b *normal microscopy using transmitted light (cells with dark outline appear a bright lemon yellow fluorescent colour by fluorescence microscopy due to the presence of the alkaloid berberine which is difficult to define by normal microscopy)*

distinguished as diffused spots of blue fluorescence that are not associated with the plant structure.

Recent medical applications may have far-reaching results. One of the most noteworthy arises from the preparation of fluorescent conjugates with proteins. A. H. COONS and M. H. KAPLAN[25] have shown that it is possible to localize an antigen in tissue cells. The antibody solution of high titre is conjugated with fluorescein *iso*cyanate and the resultant fluorescent antibody solution is employed as a specific histochemical stain on tissue sections. Wherever antigen–antibody precipitates are formed, the fluorescein-antibody is fixed. Fluorescent proteins which have not reacted are washed away and the section is examined by fluorescence microscopy. The deposited fluorescein-antibody conjugate can be identified by its yellow-green fluorescence, and reveals the presence and location of the homologous antigen.

B. A. NEWTON[26,27] has used the principle of forming fluorescent conjugates in studying the mode of action of polymyxin. When polymyxin is added to washed cell suspensions of *Pseudomonas aeruginosa*, certain soluble constituents of the cells leak through the cell walls. The bacterial activity of this antibiotic may be due to its ability to combine with certain chemical groups at the cell surface, resulting in disorganization of the cell membrane or osmotic barrier. A. V. FEW and J. H. SCHULMAN[28] have described the same action with other organisms. Newton showed that cells can be protected against the bacterial activity of polymyxin by certain cations which compete with the antibiotic for sites on the cells[27]. G. WEBER[29] used N-tolylnaphthylamine-8-sulphonic acid (T.N.S.) to form fluorescent protein conjugates. Washed cell suspensions of *Ps. aeruginosa* treated with T.N.S. do not fluoresce but, in the presence of polymyxin, fluorescence in the cells develops rapidly, demonstrating that T.N.S. can penetrate to the protein-containing portions of the cell which form fluorescent conjugates. B. CAMBER* has recently demonstrated the use of salicylic acid hydrazide as a microchemical reagent for visual differentiation of aldehydes and ketones by fluorescence. This technique has been applied to the biological staining of tissues containing ketosteroids.

The experimental sections are compiled from work undertaken in the Government Laboratory. The author's thanks are extended to a number of colleagues who have cooperated, and to the Government Chemist for permission to publish this article.

* Demonstration at the 333rd Meeting of the Biochemical Society, London, October 1954.

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NUMERICAL VALUES AND QUANTITATIVE DETERMINATIONS

in microscopy

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The microscopical determination of numerical values is primarily applicable to vegetable matter. Such values are often criteria (e.g. *palisade ratio, stomatal index*) *which characterize certain substances, particularly powders. Some accessory apparatus must be fitted to the microscope for determinations of this type.*

THE SIMPLEST numerical values determined by the use of the microscope are measurements of thickness, refractive indices (by the immersion method), blood counts and counts of bacteria in vaccines. All these values are obtained by well established methods and need not be described here. One feature, however, becomes evident, namely that, except for refractive indices and measurements of thickness, there must in general be some structural objects to be counted, in order to obtain a numerical value characteristic of the material in which the observed structures occur. It is therefore chiefly in relation to vegetable products that microscopically determined numerical values can be used. Many of these values are criteria by which substances can be identified and are more particularly useful for the characterization of powders.

APPARATUS

Certain accessory apparatus is necessary for almost all determinations of this type. Some arrangement for making drawings is frequently required, and ruled gratings and scales are needed to drop into the eyepiece, sometimes also a scale to be used as an object on the stage of the microscope. Two of the simplest diagnostic criteria that can be obtained are (*a*) the palisade ratio and (*b*) the stomatal index, both of these being important values for the identification of leaves, to which they can be applied either in the unground condition or in the form of powder. In these two determinations apparatus for making drawings, such as a camera lucida, is essential. Generally, the microscopical preparations used will be temporary mounts in fluid media and this necessitates the use of a microscope in a vertical position with the stage horizontal. The type of camera lucida must therefore be selected so as to conform with this requirement.

Camera lucida—The Abbe camera lucida and others based on the same principle, such as the Swift-Ives pattern, fulfil these requirements. The Abbe camera lucida consists of a prism fitted over the eyepiece of the microscope and, attached to the casing of the prism, is an arm carrying a plane mirror which is supported vertically over the drawing

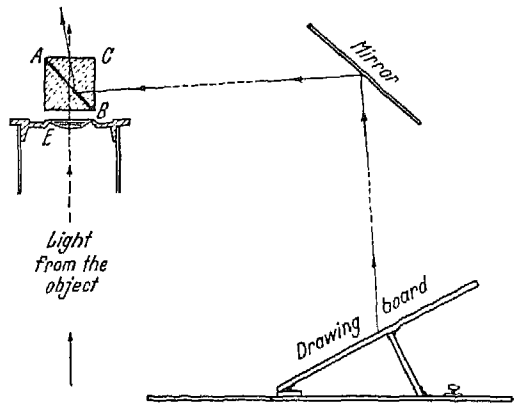

Figure 1. Abbe camera lucida with drawing board: ACB prism over the microscope eyepiece ; AB silvered surface of the prism, with a central hole ; E eye-lens of the microscope eyepiece

paper. The prism consists of a cube of glass cut diagonally to form two right-angled prisms and the face AB of the upper prism ABC (see *Figure 1*) is silvered with the exception of a small oval hole at its centre. When in use, light from the drawing paper is reflected by the plane mirror into the prism *ABC*, and is then reflected upwards through the face *AC* into the observer's eye who will therefore see the drawing paper in the direction of the stage of the microscope. At the same time, the microscopist sees the image of the object in the microscope through the small hole and can trace its outline on the drawing paper.

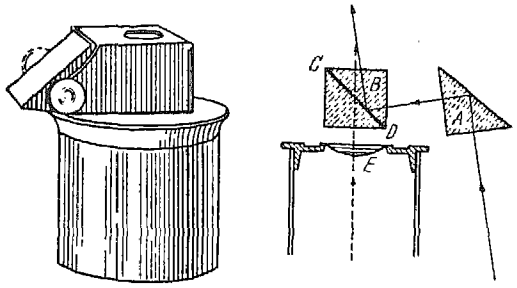

Figure 2. Swift-Ives pattern camera lucida: A movable, right-angled prism to receive light from the drawing board and reflect it into the prism B, from the silvered surface CD, of which it is reflected upwards into the observer's eye; E eye-lens of the microscope eyepiece (the vertical dotted line represents light coming from the image in the microscope and passing through the central hole in CD to the observer's eye)

Before this is done, the drawing-board must be adjusted to give uniform magnification over its surface; to obtain this result a scale, termed a stage micrometer, is necessary. This micrometer consists of a 3 in. by 1 in. slide on which a scale (usually 1·1 mm long), divided into tenths and hundredths of a millimetre, is either engraved or made by photography. This scale is placed on the stage and focused by the microscope when the observer can trace the lines of the scale upon the drawing paper. The scale of the stage micrometer is usually divided into tenths of a millimetre and the terminal tenth is further sub-divided into hundredths. The drawing board, on which the paper is fastened, must be tilted by raising the edge furthest from the microscope until the divisions drawn on all parts of the board are equally spaced; at this inclination drawings and measurements can be accurately made at a known magnification.

The magnification is determined by measuring the distance between selected lines on the drawing, and dividing by the distance between the corresponding lines of the stage micrometer. The Swift-Ives camera lucida resembles the Abbe pattern, but the plane mirror is replaced by a small right-angled glass prism (*Figure 2*).

PALISADE RATIO AND STOMATAL INDEX

The two simple values, termed ' palisade ratio '[1] and ' stomatal index '[2], are based upon the structure of leaves. Leaves have an upper and a lower epidermis, both of which are composed of tabular cells in a single layer, and beneath the upper epidermis there is usually a layer of cylindrical cells arranged with their long axes at right angles to the epidermis, as can be seen in a transverse section of the lamina of the leaf (see *Figure 3C*); these are the palisade cells. When, therefore, the cleared leaf is examined in surface view, the palisade layer is seen as rather smaller circular cells under the epidermis and the average number of palisade cells present under one

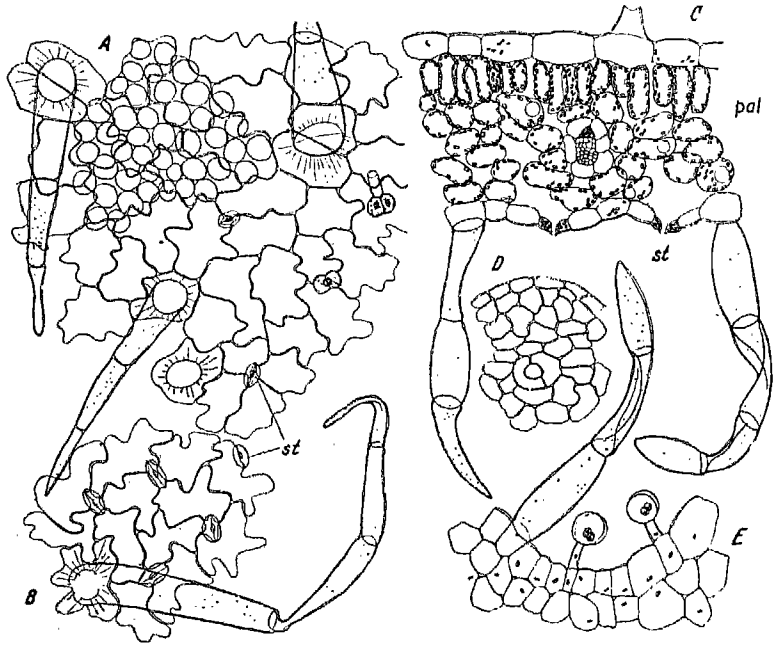

Figure 3. Leaf of foxglove (Digitalis purpurea *L.*): *A upper epidermis with underlying palisade cells, showing the palisade ratio; B, lower epidermis with stomata, st.; C transverse section of the leaf blade, showing the palisade layer, pal., and the two epidermises* (×145)

epidermal cell is known as the ' palisade ratio ' (see *Figure 3A*).

The epidermis itself on the underside of the leaf, and sometimes on the upper side also, consists of two kinds of cells resulting from the presence of openings termed stomata (each is a stoma) to allow air to reach the tissues of the interior of the leaf. Each stoma is composed of two cells, named ' guard cells ', and these differ in shape and size from the ordinary tabular epidermal cells. The stomatal index is the percentage which the number of stomata forms of the total number of epidermal cells, including the stomata, each stoma being counted as one cell.

Both these values are fairly constant for the leaf of any particular plant and can be used as two of the more important characteristics by which the botanical origin of a leaf can be established[3]. Their determination is quite simple, because they are both ratios and do not involve weighing.

Palisade ratio—For the palisade ratio, a count is made of the number of palisade cells under four adjacent epidermal cells and the count is repeated for a total of five groups of four cells, each in different parts of the leaf. The average of the results for the five groups is taken as the palisade ratio for the leaf.

For the determination an approximately square piece of leaf, about 5 mm edge, is heated in a test tube with solution of chloral hydrate (5 parts of chloral hydrate and 2 parts of water) by supporting it in a boiling water-bath for about 10 to 15 min. This treatment renders the leaf fragment transparent. It is then transferred to a microscope slide and mounted, upper epidermis uppermost, in the solution of chloral hydrate (it is advisable to put a small drop of glycerol on one side of the cover glass to prevent the preparation from drying). The slide is examined with a 4 mm objective and a $\times 6$ eyepiece, to which a camera lucida is attached. Four adjacent epidermal cells are traced; gentle downward focusing then brings the palisade into view and sufficient palisade cells are traced to cover the area of the outlines of the four epidermal cells (see *Figure 3A*). This operation is repeated for five different groups of four cells each and the final result expressed as a range with an average, for example *Digitalis purpurea* L. 3·5 to *4·0* to 4·2.

Stomatal Index—This is a second value used for the identification of leaves[4]. The simplest procedure is to focus with a 4 mm objective the epidermis of the cleared leaf and, using a camera lucida, mark on the drawing paper a cross (x) for each epidermal cell and a circle (o) for each stoma. The number of crosses *E*, *i.e.* epidermal cells, and the number of circles *S*, *i.e.* stomata, are counted and the stomatal index calculated from the formula $[S \div (E + S)] \times 100$. This experiment is repeated for ten fields selected on a plan such as that suggested below and illustrated in *Figure 4*. The ten fields are selected by moving the slide on the stage so as to bring the positions, marked with large figures on the diagram, successively under the objective; the figures indicate distances in millimetres from the centre of the preparation. To move the slide a mechanical

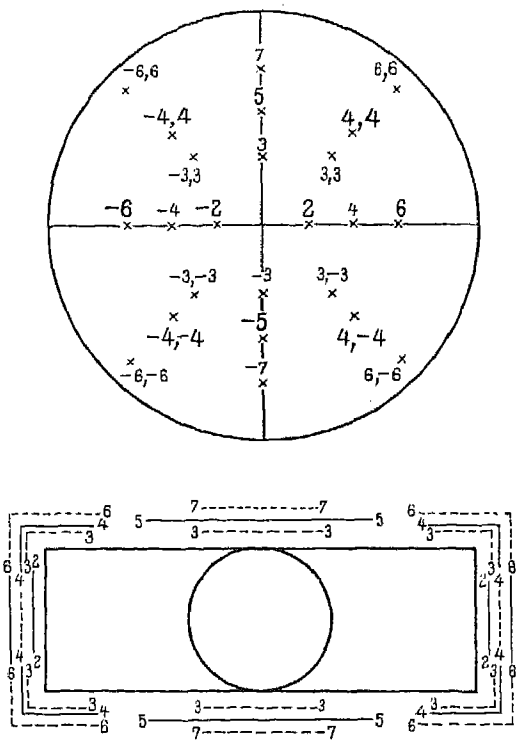

Figure 4. The upper diagram shows the position of ten selected fields, indicated by the larger numerals, and fifteen other selected fields represented by the smaller numerals, together with the centre of the circle; the lower diagram is a counting-field finder, corresponding to the selection of fields shown in the upper diagram

stage with graduated movements may be used. It is, however, simpler to dispense with the mechanical stage and to replace it by a card, termed a ' counting-field finder '[5]. The ' finder ' is made of thin cardboard upon which is drawn a rectangle 3 in. by 1 in.

Parallel to the sides of this rectangle and outside it, lines are drawn at distances in millimetres

corresponding to the positions of the fields marked in *Figure 4*. The distances of the lines from the sides of the rectangle are indicated by the figures against them and the ten unbroken lines correspond to the fields marked by the larger numerals, while the fourteen broken lines correspond to the fourteen positions marked by the smaller numerals. The finder can be attached to the plain stage of the microscope by piercing it with the pins of the clips on the stage or by the use of an adhesive or some kind of clip.

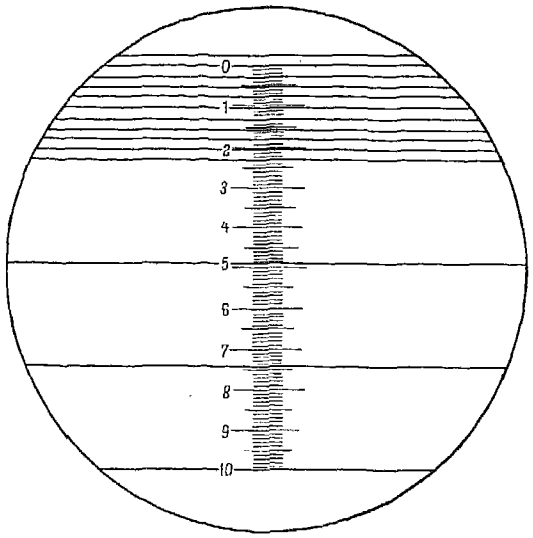

Figure 5. Drawing of the eyepiece and stage micrometers as seen in the eyepiece of a microscope, using a 4 mm objective and a ×6 eyepiece—100 scale divisions of the eyepiece micrometer, numbered 1 to 10, are seen to be exactly equal to 3·9 divisions of the stage micrometer i.e. *to 0·39 mm; one small eyepiece division therefore equals 3·9 μ*

OTHER SIMPLE NUMERICAL VALUES

Linear measurements—Linear measurements can be made from the drawings described above, by measuring the required length in millimetres, dividing by the magnification and expressing the result in microns. More usually, however, these measurements are made by means of a scale dropped into the eyepiece of the microscope and supported on the diaphragm that is fixed between the two lenses of a Huyghens eyepiece. It is at the level of this diaphragm that the real image is formed in the microscope and consequently the image and the scale are coincident; the length of any structure in the image can therefore be read off immediately in terms of scale divisions. The scale is calibrated by placing the stage micrometer on the stage of the microscope and focusing the lines which will be seen in the eyepiece with the eyepiece-scale superimposed. The number of divisions of the stage micrometer which are equal to 100 eyepiece divisions is read off and the value of one eyepiece division calculated (*Figure 5*).

Area measurements—Area measurements for particles of geometrical shape can be determined by calculation from their linear dimensions. For irregular particles, which are those most commonly found, the outline must be traced at a definite magnification and the area of the drawing measured by carefully cutting out the piece of paper and weighing it, by making the drawing on squared paper and counting the squares enclosed by the outline, or by using a planimeter. In the author's experience, the most generally useful method is to cut out and weigh the piece or pieces of paper and calculate the area from the weight of unit area of the same paper. This method gives a slightly better result than the planimeter, but not quite so good as that obtained by counting the squares of millimetre squared paper. This counting of squares is very tedious and does not offer a compensating advantage in accuracy over the weighing method. The area thus found is divided by the square of the magnification of the drawing and the result is expressed in any convenient terms.

Cells per unit area—A useful qualitative diagnostic characteristic, based on number and area, is a count of the number of cells of a certain type per unit area of a tissue. This has proved particularly valuable in the characterization of several closely related species of plants, which yield the various types of commercial cardamoms. Cardamom seeds contain a layer, one cell thick, of beaker-shaped thick-walled (sclerenchymatous) cells and portions of this layer are easily found in the powered spice. To make a count, a net-ruled disk is dropped on to the diaphragm of the microscope eyepiece and the draw-tube of the microscope is adjusted until the side of each small square corresponds to 0·0317 mm of a scale placed on the stage of the microscope. Each small square will then correspond to 0·001 mm^2 of any object on the stage. Using a camera lucida, the outline of ten adjacent squares lying over a fragment of the sclerenchymatous layer is traced; a cross is made for each cell included within the ten squares and the crosses counted. This number multiplied by 100 is the number of cells per square millimetre for that fragment; this should be repeated for thirty fragments which will give a result correct to 5 per cent. *Figure 6* illustrates the differences in the number of cells per square millimetre for three kinds of cardamom[6].

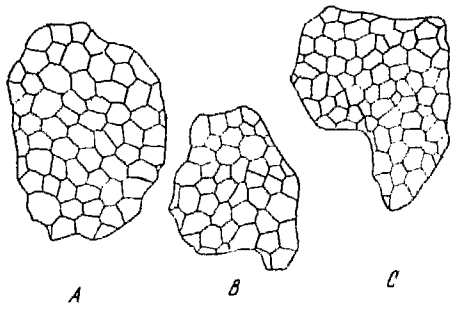

Figure 6. Outlines of cells in fragments of the sclerenchymatous layer of three varieties of cardamom seeds (×175): A Aleppi (3920 cells/mm²); B Malabar (4700 cells/mm²); C Korarima (5900 cells/mm²)

QUANTITATIVE MICROSCOPY

It is desirable to apply the term 'quantitative microscopy' to the determination of the mass (or weight) of any substance seen in the field of the microscope. This limitation would conform to the use of the term 'quantitative' in chemical work and would exclude from this category the making of simple linear measurements and determinations of area.

Since the microscope is merely an aid to vision, it is impossible to make weighings directly. The weight, or mass, of any substance must be estimated by counting particles which must be uniform in size and of which the average mass is known, or—with suitable materials—the superficial areas can be measured of portions which have a definite thickness and a known density.

The method based on measurement of area has been in use for more than a century and was introduced by petrologists for the determination of the proportions by weight of the different minerals present in rocks. A section of known thickness is prepared from the rock and is mounted on a microscope slide; the areas of the various pieces of minerals present are traced to scale by means of a camera lucida and the areas are measured by one of the methods referred to earlier. The volumes are calculated from the known thickness and the weight is obtained by multiplying by the density. By this means microscopical methods have been applied to the determination of the constituents of rocks[7], and to the examination of granites[8], of paving sets[9], and of cements and concretes[10].

For the quantitative determination of the proportions of the constituents of mixed powders, a rough estimate can be made for some of the simpler types of mixtures by comparing the appearance of mounts of the material of unknown composition with mounts of admixtures of known proportions of the substances found to be present. This procedure is useful in that it provides a general idea of proportions, but is not sufficiently precise and impartial for the assessment of the amounts in numerical terms, which could be regarded as accurate within certain specified limits. The observer's judgement is the only governing factor and will obviously vary between different microscopists, reducing the result to the status of a personal opinion.

Several attempts have been made to devise apparatus with which accurate numerical results might be obtained. Such apparatus has required the use of either a specially constructed mechanical stage or a specially designed slide, but has never come into general use. What is required is a process which can be used on any microscope with any convenient combination of lenses without the addition of special accessories. This has been effected by the 'lycopodium method'[11,12].

LYCOPODIUM

Lycopodium is a natural product consisting of the spores of the club-moss, *Lycopodium clavatum* Linn., and is a regular article of commerce, finding its greatest use in the manufacture of certain kinds of fireworks. In its commercial form, lycopodium is a very light, mobile, yellow powder, the moisture content of which is about 5 per cent and varies so little in different samples that it has no disturbing effect upon the experimental results obtained by its use.

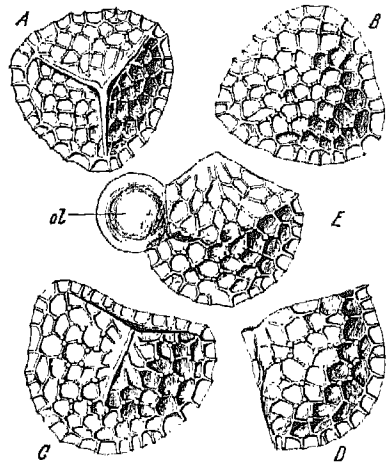

Figure 7. Lycopodium powder—the spores of Lycopodium clavatum *L. (×800): A spore seen from above to show the apex; B base of a spore; C spore partly turned over; D side view of a spore; E spore burst by pressure on the cover glass to show a globule of fixed oil, ol, emerging from the split*

When examined microscopically, the powder is seen to consist of very characteristic and extremely uniform spores. Each spore is tetrahedral and has the form of one quarter of a sphere; this results from their formation in the sporangium of the plant, four spores being produced by the division of one spherical mother-cell, the dividing walls all meeting at the centre of the sphere. Each spore therefore has a rounded base corresponding to the spherical surface of the mother-cell and three flat triangular faces, where it was in contact with its neighbours in the same mother-cell, these faces meeting in three straight ridges, which themselves meet at the apex of the spore (*Figure 7*). It is evident, therefore, that the length of one of the straight edges of the spore is the radius of the spherical mother-cell and, if this length is measured, the volume of four lycopodium spores can immediately be calculated. The average of a number of measurements, which vary little amongst themselves, is 21 μ. Thus the volume of one lycopodium spore is $0{\cdot}97\times10^{-8}$ cm^3 and the specific gravity of lycopodium is 1·086, so that the weight of one spore is $0{\cdot}1053\times10^{-4}$ mg. The number of spores per milligram of the powder is therefore $1/0{\cdot}00001053=94{,}970$. The experimental error in this determination does not allow any reliance upon figures less than thousands, and the result is therefore recorded as 95,000 spores per milligram.

The number of spores per milligram of lycopodium can also be found by direct counting[12]. For this purpose a count can be made of the spores in a weighed amount of a suspension and the following is an example of this determination. About 0·1 gm lycopodium is accurately weighed and mixed with 8 to 10 ml olive oil or of a suspending agent consisting of 2 vol. of glycerol, 1 vol. of mucilage of tragacanth* and 2 vol. of water. The required amount of suspending agent is put into a small conical measure; the weighed lycopodium is transferred to a glass plate about 15 cm square, thoroughly mixed with a few drops of the oil or glycerol–mucilage by means of a flexible spatula and gradually incorporated with sufficient of the fluid to make a thin paste. The suspension is drained off from a corner of the plate into a clean and dry tared weighing bottle. The residue on the plate is rubbed with further small amounts of the oil or glycerol–mucilage and drained into the weighing bottle until the whole of the powder has been transferred. The remainder of the suspending agent is added to the bottle and the whole is mixed by gentle oscillation. The bottle and contents are weighed, and the weight of the suspension found. A clean microscope slide and cover glass are weighed, a drop of the suspension is placed on the centre of the slide, the cover glass applied and the slide weighed again, giving the weight of suspension under the cover glass.

Using a counting-field finder or a mechanical stage, the number of spores is counted in twenty or twenty five fields, selected as explained earlier. The diameter of the field of view is measured and its area calculated. From the area of the cover glass and the number of spores in the twenty to twenty five fields, the number of spores under the cover glass is found. From the weight of suspension under the cover glass the weight of lycopodium present is found, and from these figures the number of spores per milligram of lycopodium is calculated. An example will make the method clear:

Weight of lycopodium	=0·1092 gm
Weight of suspension	=11·5814 gm
Weight of suspension on the slide	=0·0132 gm
Number of spores in 20 fields of view	=8, 7, 4, 6, 8, 10, 5, 8, 4, 6, 7, 13, 10, 12, 6, 11, 6, 13, 4, 10=158 spores
Area of one field	=0·2003 mm^2
Area of 20 fields	=4·006 mm^2
Area of the cover glass	=298·7 mm^2
Number of spores under the cover glass	$=158\times298{\cdot}7\div4{\cdot}006=$ 11,780 spores
Weight of lycopodium under the cover glass	$=13{\cdot}2\times0{\cdot}1092\div$ 11·5814 gm
Number of spores per mg of lycopodium	$=(11{,}780\times11{\cdot}5814)\div(13{\cdot}2\times0{\cdot}1092)=94{,}640$ spores

The mean of twenty six determinations made on four samples of lycopodium was 93,000 and the average from the two methods of experiment gives 94,000 spores per milligram of air-dry lycopodium. It is now possible to determine accurately the weight of any number of spores counted in a field of view of the microscope.

Not only is lycopodium composed of uniform particles, but the spores are also very easily identified because of their characteristic shape and surface markings (*Figure 7*). They are also remarkably strong and do not break when triturated as described previously. In addition they remain unaltered when treated with the ordinary microscopical mountants, such as chloral hydrate solution, glycerol *etc* and they are not affected by dilute acids and alkalis or by strong hydrochloric acid[12].

For counts of particles by means of the microscope, it is necessary to use a small glass disk, usually known as a squared or net-ruled micrometer. This disk fits into the Huyghens eyepiece and rests on the diaphragm; it has a central area of 1 cm^2 ruled

* Mucilage of tragacanth: Take 1·25 gm finely powdered tragacanth and add to it in a dry vessel 2·5 ml industrial alcohol (97 per cent); mix and add rapidly, with constant stirring, 100 ml distilled water.

into 100 small squares, each 1 mm^2 in area. The rulings are focused, if necessary, by adjusting the eye-lens of the eyepiece, and the count of particles in the field is made by counting the number in each small square in succession until the whole area of 1 cm^2 has been covered.

DETERMINATION OF MASS

If any powdered substance is mixed with a definite proportion by weight of lycopodium and if a microscopical mount is prepared from a suspension made of the mixture, the weight of lycopodium seen in the field of the microscope can be found by counting the spores, and from the proportions in the mixture the weight of the other substance is obtained. If this second substance contains any characteristic countable particles, such as starch, hairs, pollen grains, stone cells *etc*, the number of such characteristic particles per milligram of the substance can be determined. In this way standard values are obtained to represent many different materials.

There are four types of particles in powdered materials which may be presented for counting, and they require different means of assessment according to their nature:

(*1*) small discrete particles, such as starch granules and pollen grains can be counted and the number forms a measure of the amount present

(*2*) linear particles, such as the fibres of cinnamon bark, or the rods of thickening in the trichomes of nux vomica, can be measured to determine their length and the total length gives an estimate of the quantity of the material which is present

(*3*) flat, plate-like particles, such as fragments of epidermis or of a sclerenchymatous layer one cell thick, as found in linseed and cardamom, provide a criterion based on the total area of such particles per milligram

(*4*) three dimensional masses of cells, such as occur in pimento, olive stones and coconut shells, require a volume determination in terms of the average cell present in the masses; this is obtained by counting for each mass the number of cells along two axes at right angles to one another, and taking the average as the diameter of an imaginary sphere representing each mass (see *Figure 10*): the volume of each sphere determined from this diameter gives the number of cells in the mass and the total number of cells per milligram of the substance is the criterion required.

Examples, giving fairly complete details of the experiments, will help to explain the *modus operandi* for these different types of particles.

Type 1—Cornflour or commercial maize starch (see *Figure 8*), is a good example of a substance consisting of discrete particles. To determine the number of starch granules per milligram, 0·2 gm lycopodium was mixed with 0·1 gm maize starch and the mixture suspended in about 20 ml olive oil. Four slides were prepared and ten fields were

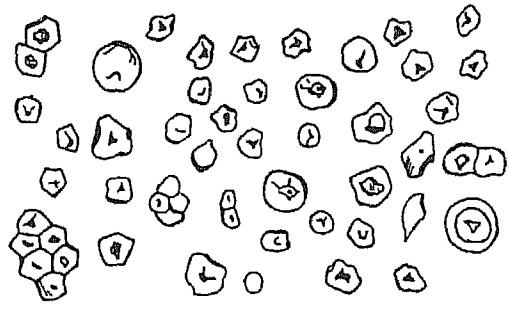

Figure 8. Starch of maize, Zea mays *L.; the angular granules are from the hard yellow part of the endosperm and the rounded ones are from the inner, white, mealy part (×286)*

counted on each slide, using a 4 mm objective. The first slide gave in the ten fields, 142 spores of lycopodium and 682 starch granules *i.e.* 480 starch granules for 100 lycopodium spores; the other three slides gave 477, 436 and 410 starch granules respectively for 100 lycopodium spores; the average for the four slides is therefore 450 starch granules for every 100 lycopodium spores[11]. Hence there are $450 \times 94{,}000 \div 100$ starch granules for every milligram of lycopodium and, since 1 mg lycopodium was mixed with 0·5 mg starch, there are $2 \times 450 \times 94{,}000 \div 100 = 846{,}000$ granules per mg of air dried maize starch.

Note: When counting starch granules, all particles distinctly recognizable as such are counted, minute specks having no definite form or character are omitted.

Type 2—Powdered cinnamon bark affords a good example of length measurement. This bark contains numerous phloem fibres (*Figure 9*), and the total length of fibre per gram is a useful means of determining the amount of cinnamon in admixture with other ingredients which might be other spices in a mixed spice or might be some adulterant, such as cassia bark.

Weighed amounts of cinnamon bark, say 0·1 gm, and of lycopodium, say 0·05 gm, are cleared by heating them on a water-bath with a solution of chloral hydrate (5 parts chloral hydrate and 2 parts water). An effective method is to rub down the mixed powders on a glass plate with some of the chloral solution (using about 3 ml altogether) to a smooth paste; the mixture is transferred to a

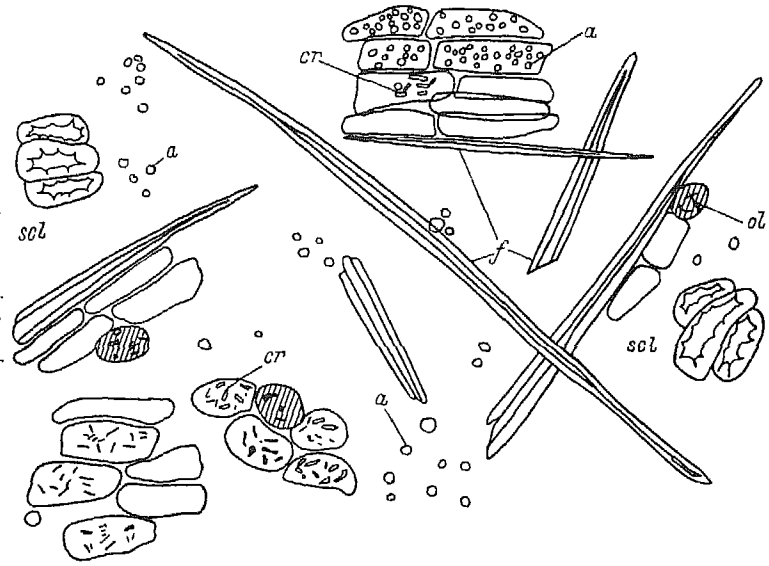

Figure 9. Powdered cinnamon—bark of Cinnamomum zeylanicum *Nees: a starch granules; cr small rod-shaped (or needles) of calcium oxalate; f fibres; ol volatile oil (partly resinified); scl sclereids from the pericycle; the total length of fibres per gram is measured and affords an excellent criterion in the determination of the proportion of cinnamon in any mixture (× 145)*

small corked tube that is lowered by a string into a boiling tube which is then immersed in the boiling water of the bath. After about 5 min the powder becomes cleared and a suspending agent is added till the volume is about 7 ml. The suspending agent is composed of glycerol (2 vol.), mucilage of tragacanth (1 vol.) and water (2 vol.). After thorough mixing, four drops of the suspension—one drop on each of four slides—are mounted and square cover glasses, about 19 mm edge, applied. For the measurements five strips are used across the cover glass, each having a width equal to the diameter of the field of view of a 4 mm objective and separated from each other by intervals of 2 mm. Using a camera lucida, the lengths of the images of all the fibres and portions of fibres seen in the five strips are marked by lines. The magnification of the drawings is found; the sum of their lengths is determined and divided by the magnification, which gives the actual length of the fibres.

The average number of lycopodium spores in one field is found by counting them in twenty four fields selected by the counting-field finder; then the number of spores in the five strips is calculated. From the total length of fibres and the number of spores present, and the proportions of the two by weight in the suspension, the total length of fibres per gram of the cinnamon is calculated. On an average this amounts to 287 ± 10 metres per gram of the bark dried at 100°C[13].

Powdered cassia bark is very similar in general structure to cinnamon bark, but it contains a much smaller proportion of phloem fibres. The average total length of fibres in cassia is 44 ± 2 metres per gram, which is between one sixth and one seventh of the amount in cinnamon. It is therefore possible to calculate with considerable accuracy the proportions of cassia and cinnamon in a mixture of the two barks, a result which is particularly useful when cassia has been used to adulterate cinnamon.

Type 3—Area measurements per gram of a substance are particularly useful for the determination of the amount of a leafy commodity present in mixtures. The characters of the epidermis are in general markedly diagnostic of the structure to which it belongs, and can usually be recognized in mixtures with accuracy. To determine the area of epidermis per unit weight, the powdered leaf is mixed with a known proportion of lycopodium, a known weight of the mixture is cleared, usually by warming with solution of chloral hydrate (as described before), and is then suspended in the glycerol–tragacanth medium. The pieces of leaf epidermis in ten or twenty fields, selected as explained previously, are traced with a camera lucida at a definite magnification, the pieces of paper are cut out, and their area determined by weighing. The area thus obtained is divided by the square of the magnification, giving the actual area of epidermis observed. From the count of lycopodium spores in the same fields, the weight of powder examined is obtained, and the area of epidermis per milligram is calculated.

For many leaves, the microscopical work on the powder can be checked by determining the area and

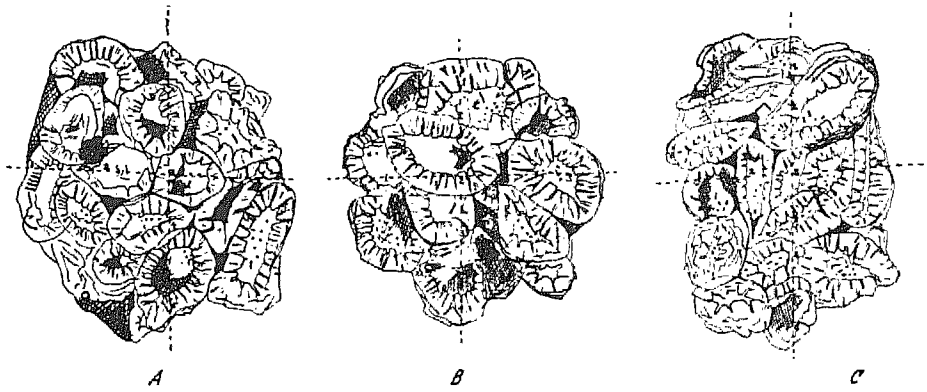

Figure 10. Sclereids from the pericarp of pimento (or allspice), the fruit of Pimenta officinalis *Lindl.: A, B and C, three typical masses of sclereids from the pericarp of pimento ; the dotted lines are the diameters across which the numbers of cells were counted in calculating the radius of the imaginary equivalent spheres*

weight of a number of entire leaves. The results obtained from entire leaves are always slightly greater than those found for their powders, because a certain small amount of epidermis is unavoidably destroyed during the powdering process, amounting on an average to about 12 per cent, but varying according to the method of comminution.

Type 4—Some plant members contain thick-walled, pitted and lignified cells in groups, which are more or less ovoid in shape. Groups of this kind are characteristic of the pericarp of pimento or allspice, the fruit of *Pimenta officinalis* Lindley. It is not possible actually to count the cells in these masses, but they can be broken into their component cells by treatment with an oxidizing agent, such as nitric acid, which will dissolve the middle lamellae and liberate the individual cells. This method is not suitable for routine use, and it has been shown, by comparison with the disintegration method, that the number of cells can be determined by calculating the volume of an imaginary equivalent sphere, the diameter of which is expressed in terms of the average number of cells obtained by counting the number of cells along two lines at right angles to one another across the cell mass (see *Figure 10*).

The volume calculated from this diameter gives the number of cells in the mass and the result is reliable, provided that not less than twelve particles are counted[14]. Proceeding in this way, it has been shown that there are 3577 ($\pm$6 per cent) sclereids per milligram of powdered pimento and this value can be used to determine the proportion of pimento present in mixtures of spices.

It has been found that the same convention can be successfully applied to powdered olive stones[15] or powdered coconut shells since, on powdering, these materials break up into irregular masses associated with a proportion of isolated cells. The cells in the irregular masses can be counted by using the device of the equivalent sphere, provided that not less than twelve masses are used for each count. The isolated cells in the same fields are also counted, and are added to the results obtained from the masses. The value obtained for olive stones is 15,140 ($\pm$6 per cent) sclereids per milligram.

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SURFACE MICRO-INTERFEROMETRY

Recent developments and description of new interferometer

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The evolution of surface interferometers is broadly surveyed on a functional basis, from the simplest unaided-vision forms to the most recent micro-interferometer developments. Surface interferometers depending upon simple (double beam) interference are considered in two classes according as to whether the interfering beams have a common path or a divided path on the object side of an amplitude dividing beam splitter. A new form of surface micro-interferometer of the second class is described.

IN RECENT years interferometry has been increasingly employed for the accurate measurement of the geometrical form and micro-structure of surfaces in terms of the wavelengths of light. In principle, the method is almost as old as Newton's description of the well known 'Newton ring' effect, and simple technical applications of this phenomenon are now very widely known. Some examples are (*a*) the use in optics of surface test plates of accurately known form and the analogous engineers' test for gauge blocks[1], (*b*) forms of interferometer microscopes[2] depending on the use of a contact plate or some more complex form of amplitude divider in the vicinity of the anterior focal plane of the objective of a microscope with vertical illumination, and (*c*) the Twyman-Green interferometer[3] which has both compensated and uncompensated forms, and can be applied (and indeed finds its widest application) to transparent test objects and variational optical path difference measurements, which are however beyond the scope of this article.

These forms are represented schematically in *Figures 1A*, *1B* and *1C*. In the former two cases the interfering beams, before and after reflection at the reference surface and the surface under test respectively, traverse a common path, division of amplitude generally taking place in the vicinity of the surface under test. Surface interferometers of this type will here be referred to as class 1. *Figure 1C* represents a form in which the interfering beams for the most part traverse separate and independent paths, an independent reference beam being produced by a semi-reflecting beam splitter immediately after collimation. *Figure 1E* represents the lesser known contemporary type of Twyman-Green interferometer[3] which may similarly be regarded as an alternative form of surface interferometer of the same class, similarly characterized by the divided path and independent reference beam. Instruments of this type will here be referred to as class 2.

It has been recognized that, in view of the emphasis in recent technical applications on the need for high magnification and increased resolution, the determining optical conditions favour instruments of class 2 in which the beam splitter does not place restrictive demands on the front working distance of the objective and thereby limit its power. In fact, the more important recent developments, commencing with Linnik's interferometer, relate to instruments of this class.

MERITS AND DISADVANTAGES OF CLASS 1 INSTRUMENTS

At an early stage in the development of Linnik's interferometer it was also recognized[4] that the need for pairing the microscope objectives used, so that they should have identical retardation properties over a range of wavelengths, imposed considerable difficulties in the practical realization of such instruments. Efforts have been made, largely on account of this, to develop instruments of class 1 requiring only one objective common to the two interfering beams.

The instrument represented in *Figure 1B*, which is in principle derived from that of *Figure 1A* by the simple addition of a microscope with vertical illumination, is an uncompensated interferometer strictly only usable in monochromatic light. In this, the reference beam reflected from the comparison plate always differs in phase from the beam reflected from the specimen under test by comparable amounts of the same sign, which results in a serious limitation to the usefulness of the instrument. By interposing a thin compensated beam splitter exactly midway between the anterior focal plane and the plane front face of the objective (made reflecting over a small central area), A. H. MIRAU[5] converted the simple form shown in *Figure 1B* into a compensated form with freedom of adjustment permitting positive and negative phase differences. In this instrument the working beam is reflected from the surface under

test in the normal manner, while the reference beam, after an intermediate reflection at the beam splitter, is focused on the front objective face and returns to trace a path identical with that of the beam reflected from the test surface. The limitation in power of objective and in numerical aperture, which is inherent in any system incorporating an additional element between objective and object, still prevents the application of this instrument for higher powers.

Interference microscopes of similar form but fundamentally different in conception have been devised to enable small path differences to be measured with greater accuracy. Use of the principle of multiple reflection[6] between test surface and reference surface, whereby the resulting interference intensity distribution is converted from the cosine squared type into the Airy type and consequently much sharper fringes are produced, has led to the design of simple instruments[7] which introduce into engineering techniques an accuracy unprecedented in the measurement of surface contour. Mention may here also be made of the analogous application of the Fabry-Perot mirror-pair by A. M. FREDERIKSE[8], improved by T. MERTON[9] by the addition of an aperture-selective zone plate enabling increased numerical aperture to be obtained. However, the optical path difference measurable in this way is seriously limited. J. DYSON[10], perceiving the difficulties caused by locating the beam splitter in the object space of the objective, abandoned vertical illumination and arranged the division of amplitude to take place in the below-stage image space of the condenser system. This system, supplemented by a unit power imaging system to extend the working distance of the objective, enables high apertures to be used. Thus, in a limited sense, the desired end is attained *viz* a compensating, single path, high power double beam interference microscope suitable for small path differences. Because it is inherently a transmission system, this type of interferometer will not be discussed in this article.

CLASS 2 INTERFEROMETERS WITH SEPARATE AND INDEPENDENT REFERENCE BEAM

W. LINNIK[11] proposed the introduction of a beam splitter into the long conjugate of a microscope objective and the use in conjunction therewith of a separate reference microscope system. He made it clear that the conception of this innovation was prompted *au fond* by analogy with Michelson's interferometer. The two instruments depend, however, upon interference phenomena which are fundamentally dissimilar and the actual form adopted[11] by Linnik differs from Michelson's by the choice of a different form of amplitude divider of the type shown in *Figure 1D*. The resulting interferometer is not free from some disadvantageous aberrational defects which affect performance, but these have been eliminated in the modified form made by Zeiss through the introduction of a collimating objective prior to the beam splitter, the actual system being that represented in *Figure 1D*.

This form of instrument may be seen to be logically related in its basic design to that shown in *Figure 1C*, just as the micro-interferometer of *Figure 1B* may be regarded as related to the visually unaided surface interferometer of *Figure 1A*. By virtue of this relation, the forms represented on the right-hand side of *Figure 1* may in general be regarded as functional derivatives of those represented on the left (without, of course, thereby implying any historical relation or allusion to their mode of origin).

The Linnik-Zeiss interferometer, *Figure 1D*, considered thus as functionally derivative of *Figure 1C*, differs from it in a manner determined by the nature of the essential purpose in view. Owing to the need—in order to produce images with increased magnification and resolution—for finite aperture in place of the negligible aperture of the narrow image-forming pencils essential to form *C*, the correlation of aperture coordinates with points in the plane of apparent location of the fringe system which is characteristic of form *C* is inevitably lost in form *D*.

This latter plane is transferred in the Linnik-Zeiss interferometer, by an interchange of the roles of aperture and field, from the field to the entrance and exit pupil planes. The instrument consequently ceases to be applicable to measurements of deformation of wave front in the pupil planes and becomes essentially a surface micro-interferometer for measuring relative retardations in the object plane.

Interferometers of class 2 with divided paths and independently adjustable interfering beams have in principle substantial advantages over those of class 1 by virtue of this independence. In addition to permitting any desired phase difference relationship between the interfering beams and unlimited power of objective to be used (if available), it also enables reference surfaces with varied and unusual reflection characteristics to be employed and allows immediate and independent control over the test beam and the reference beam. These advantages, subject to some qualifications, have made the Linnik-Zeiss interferometer well adapted, from the point of view of flexibility and ease of application, to a large number of tests now requiring to be made in engineering physics. However, the design still suffers from some important disadvantages : more optical parts

are involved than the essential optical conditions demand, and the necessity for special forms of objectives computed for an object located in the principal focal plane and long-conjugate infinity adds to the constructional difficulties.

Bearing in mind the derivative nature of the Linnik form, it may be noted that the essential functional elements (comprising two optically similar microscopes, intersecting at an amplitude divider, by means of which light from a common light source is introduced, supplying illumination for the formation of coincident images in a common focal plane) are—in an interferometrically equivalent form—broadly represented for unit magnification in *Figure 1E*.

Continuing the above *a posteriori* analysis and deduction on the basis of logical relationships distinct from the historical order, it will now be

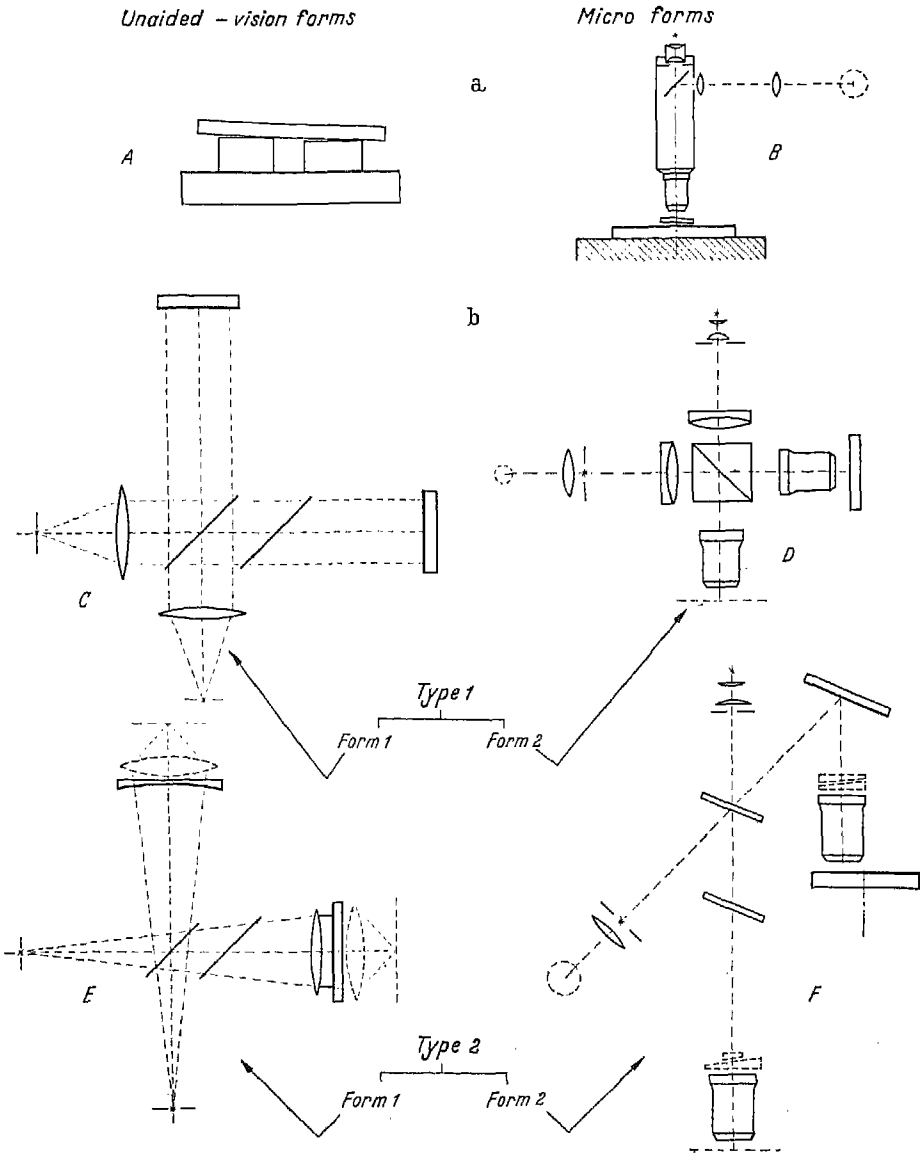

Figure 1. Surface interferometers: a *class 1—common path;* b *class 2—divided path (independent reference beam)*

demonstrated that an improved form of surface micro-interferometer related to that shown in *Figure 1E*, subject to special design conditions here indicated, may be deduced and realized.

DERIVATION OF NEW DESIGN

At first glance there would appear to be no difficulty in deriving, from the unaided-vision form of surface interferometer shown in *Figure 1E*, a corresponding form of surface micro-interferometer capable of high powers. In the course of this adaptation, interchange of field and pupil planes would of course be essentially involved, as in the case of the *a posteriori* derivation of the Linnik-Zeiss form from that in *Figure 1C*. The dotted line additions to *Figure 1E* in fact indicate substitutions which, superficially at least, have in essence the desired effect. The need for finite aperture as a *conditio sine qua non* for resolution however gives rise to serious difficulties as soon as one steps from the theoretical diagram to its attempted physical realization.

In *Figure 1E* the division of amplitude is indicated as effected by a hypothetical septum of infinitesimal thickness, but in practice this would need to be of some measurable thickness. The use of very thin pellicle membranes is here a distinct possibility, but otherwise an accurately worked plane parallel plate and a compensating plate optically identical with it are necessary. Owing however to the defect of imagery consequent upon oblique transmission of a diverging or converging beam through such a parallel plate, there normally exist, for any given point of the focal plane, appreciable differences of phase over any image-forming wave. As a result, in addition to a most undesirable loss of definition and resolution, the capacity for interference and the visibility of interference fringes produced are both considerably weakened. This can nevertheless be overcome, as will now be shown.

First, it should be noted that each component microscope system is back-reflecting so that defects of odd order are automatically eliminated. Spherical aberration and astigmatism therefore have to be considered. By the nature of the system spherical aberration may be regarded as subsidiary to astigmatism, which is known to reach considerable proportions even under ordinary conditions. In fact, the resulting spherical aberration would be so small that it need not be taken into consideration.

Considered in terms of an optical path difference δw in a given zone of semi-angular aperture u for the meridional and transverse planes, the astigmatism depends upon the thickness d of the plate, its refractive index n, a function A of the angles of initial incidence and refraction (j, j' respectively) of the principal ray, and the quantity u:

$$\delta w = \frac{u^2}{2}\left(\frac{A}{n}\right) d$$

where

$$A = \frac{1}{\cos j'}\left(1 - \frac{\cos^2 j}{\cos^2 j'}\right)$$

The astigmatism may be restricted to any chosen limit value $\delta_0 w$ by appropriate adjustment of n, j and d. If the quantity $(100\ n/A)$ is denoted by B, the following limiting value for d, assuming j and n to be known, may be derived

$$d_{\max} = \delta_0 w \left(\frac{0{\cdot}02}{u^2}\right) B$$

The quantity B may be interpreted as the thickness-astigmatism ratio for the case $u = (0{\cdot}02)^{\frac{1}{2}}$.

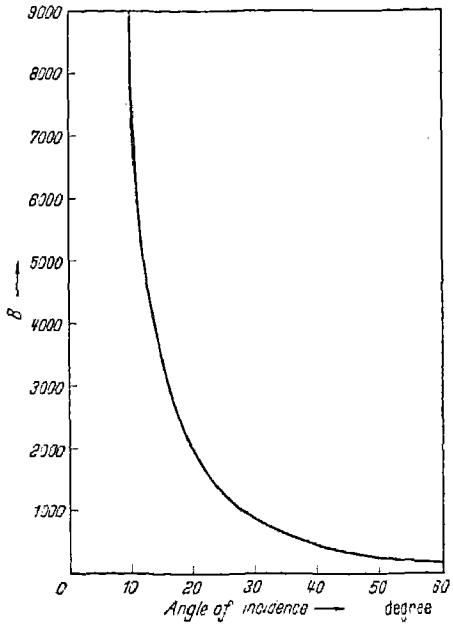

Figure 2. Relation between B and angle of incidence

Variation of refractive index affects the value of B rather inconsistently in relation to its dependence on angle of incidence, as higher values of B result from higher n values for high values of j, and from lower n values for low j values. But the difference in B over a refractive index range of 1·470 to 1·700 is nowhere greater than 10 per cent. A mean value of $n = 1{\cdot}585$ is therefore assumed. *Figure 2* represents graphically the variation of B with angle of incidence for this value of refractive index.

If $\delta_0 w$ for the margin of the aperture is assumed to be $\lambda/4$ and—taking account of an additional plate as compensator—it is assumed that the beam will traverse m times a plate of thickness d (where m may be 3 or 4 depending on whether the compensator is in contact with, or substantially separated from, the beam divider), then the value of d_{max} complying with this condition is given by :

$$d_{max}=\frac{\lambda}{4m}\frac{0{\cdot}02}{u^2}B$$

Thus, for a 4 mm objective of numerical aperture 0·72, initial magnification 40, u 0·018, and varying j values, the corresponding values of d_{max} for wavelength 5500 Å as given by this formula, assuming $m=3$, are as follows:

j :	15°	25°	35°	45°
d_{max}(mm):	10·3	3·7	1·8	1·0

There is no theoretical reason on optical grounds for adherence to the conventional value of 45° for j. From a practical point of view distinct advantages, both in robustness of the amplitude divider—as may be observed from the above values—and the ultimate form of the complete instrument, follow from adopting lower values. Angles j of 20° to 30° are suitable in this respect and the resulting general optical form is as shown in *Figure 1F*. The lower objective is that facing the surface under test, which is supported on an accurately adjustable stage. An adjustable wedge compensator situated just above the lower objective, together with its counterpart in the reference beam, has been added to the system in order to facilitate in practice the final adjustment for path length in the two component microscopes.

When such an instrument is in perfect adjustment its component microscopes behave in an identical manner in all respects and, apart from any superimposed effect deriving from the surface structure of a particular test surface, all parts of the test field focal plane will be in phase with the reference beam. It is, however, well known in interferometry that the measurement of such effects is considerably facilitated by employing a superimposed system of interference fringes resulting from a one-dimensional linear variation of path length, such as is readily achieved in the present instrument by a slight lateral, relative displacement of one of the image-forming systems with reference to the other. In order that this adjustment may be suitably controlled in relation to the effect to be measured, it must be capable of being made to micrometric accuracy in any orientation. Means for effecting adjustments of this character are incorporated in the mechanical design of the reference objective mounting.

GENERAL DESCRIPTION OF INTERFEROMETER

Figure 3 shows a general view of the instrument incorporating an optical system of the form shown in *Figure 1F*. The interferometer is built into a rigid housing supported by the main body of the instrument over the object under test. This is either mounted directly in V supports resting on a flat platform or in other forms of mounting appropriate to the geometrical form of the test object. The platform is vertically adjustable, with fine adjustment for focusing, over a range of 4 in., laterally in both coordinates by accurate 1 in. micrometers and in orientation over an angular range of $\pm 1°$ in two perpendicular planes.

Provision is also made for tests to be conducted on the surfaces of specimens which are themselves taller than the maximum of 4 in. or are integral parts of similarly large, bulky and heavy members. On loosening the clamp screws fixing the interferometer unit to the vertical column it is free to rotate and to take up a fixed position diametrically opposite to that indicated. This allows unlimited space for special tests of this kind.

The instrument is normally fitted with paired 4 mm objectives (numerical aperture 0·65) which are interchangeable with 16 mm objectives (numerical aperture 0·30). Interchangeability is secured by

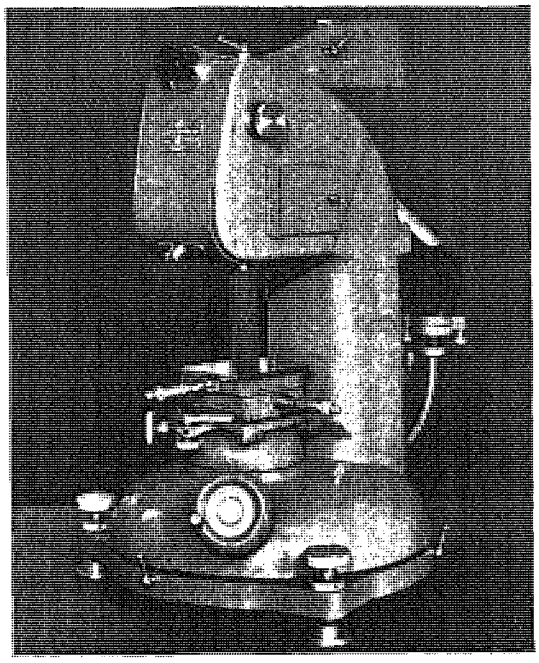

Figure 3. New Hilger and Watts interferometer

a special mechanical design of objective mounting due to C. GODFREY, depending on location by spring pressure, laterally within a cylindrical ground fitting and vertically against a fixed annular surface. The accurately controlled lateral displacement of the objective at a variable orientation for obtaining the superimposed fringe system is secured by a special design also due to Godfrey. This displacement is produced by the action of a small eccentric cam, pivoted upon an annular mount and bearing against a laterally adjustable ring in which the objective is mounted, the angular position of the cam being dependent upon the vertical position of a sliding coaxial, vertically adjustable sleeve against which bears an approximately coaxial annular plate rigidly attached to the cam. The vertical position of the sleeve and also the orientation, about the instrument axis, of the whole objective mounting are conveniently adjustable from outside the instrument, thereby providing the user with the desired form of control over the superimposed fringe system, immediately accessible during observation.

As the visibility of the fringe system depends upon the relative amplitudes of the interfering beams, an instrument such as this, applicable to test objects differing widely in reflection factor, would vary very much in its performance if no corresponding control existed over the degree of reflection at the plane reference mirror. In order to provide control of this kind, the mirror is mounted eccentrically and is metallized in two adjacent 120° sectors with evaporated aluminium and chromel respectively, giving reflection factors of approximately 95 and 40 per cent, the third sector being left unmetallized. By rotating this mirror within a mount in which it is held permanently in contact with a locating ring, the relative brightness of the reference beam can be adjusted at will according to the reflection factor of the specimen under test and high visibility maintained for all types of specimen. Light sources enabling interference to be obtained in monochromatic light (mercury radiation filtered for 5461 Å) and in white light are both mounted on the instrument and are interchangeable by simple rotation of a double arm supporting lamphouses for both sources*.

With the two interchangeable objectives, overall magnifications of 78 and 360 times using the lower power eyepiece and 162 and 750 times using the higher power eyepiece are obtained. To enable a photographic record of a test to be obtained, the instrument incorporates a camera brought into action by the rotation of a prism which is interposed near the focal plane and normally directs the image-forming beam out of the instrument at a convenient angle for observation. The photographic magnifications are respectively 78 and 360 times, the image field diameter being 61 mm and plate size 3·5 in. × 2·5 in.

* We are indebted to Messrs Siemens Electric Lamps and Supplies Ltd for producing a convenient form of their small mercury discharge lamp with approximately 5 mm electrode spacing providing a mercury source of appreciable area suitable for this instrument.

APPLICATIONS

It may appear superfluous to refer here to the possible applications of such instruments, which in scientific engineering already include measurements of the surface form, structure, finish and wear of numerous forms of surfaces, fibres *etc*—for example, gauges, bearing surfaces (plane, spherical and cylindrical) and ruled areas such as diffraction gratings. Additional possibilities exist, however, in this and other fields, depending upon special techniques.

Mention may first be made of a technique based upon that of E. ZEHENDER[12] whereby an impression is taken in a lacquer or plastic film of objects inaccessible to the instrument either for geographical reasons or because of their peculiar shape. By this means the instrument is made applicable to the interiors of tubes, cavities *etc*. The impression is taken, aluminized and presented to the instrument in the usual way. An example of the application of this technique is shown in *Figure 4* which represents the internal surface of a hollow shaft.

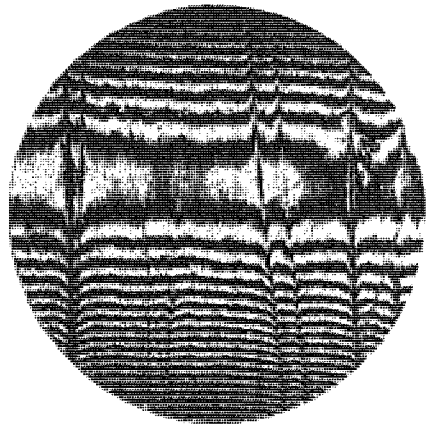

Figure 4

Micro-interferometers give, in general, contour indications of surface retardation effects. The engineering applications mentioned depend on a simple path difference measured in the axial direction between the surface under test and the theoretical image (formed in approximately the same plane by reversed imagery by the working objective) of the eyepiece focal plane image of the reference surface.

(This surface may be of any form lying within limits imposed by the field curvature of the objectives, but is in fact generally plane, in conformity with the usual requirements.) This interpretation, however, is a practical simplification of the familiar generalized path difference formula $\Delta\int_S n ds$ where n is the refractive index of the medium adjoining the surface under test. The simplification results from having homogeneous conditions in the respective media and a common value of unity for n.

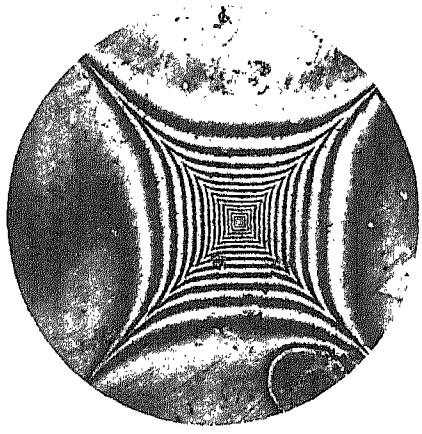

Figure 5

In biophysics the interferometer has recently been applied extensively in tests on bacteria or biological specimens embedded in a medium in contact with a plane mirror (which replaces the test object with surface structure in applications referred to above) and enclosed by a cover plate. These tests reveal structural detail due to differences in the surface distribution of refractive index, the total value of S within the medium being constant. An early application of the Linnik interferometer using this technique was made by W. KINDER[13] who described observations and measurements on red blood corpuscles.

In tests involving a special immersion medium it is clearly necessary that either n or S should be constant over the area examined unless, as in Kinder's case, supplementary steps are taken to measure and correlate refractive index differences. An important application in which n is constant and measurements are made on variations of S was described[12] by Zehender for use in conjunction with his transferable impression technique. This aims at extending the range of measurement normally available in interference microscopes, thereby enabling deeper depressions and rougher surfaces to be measured interferometrically. The measurement range is normally limited by visibility conditions to about $\pm 2\ \mu$ (*ca* $\pm 40\ \mu$ in.) or nearly double this amount photographically, depending on the spectral sensitivity of the plate. In the case of a simple test object this represents a limiting value of $n\Delta S$, where $n=1$, but in the case of an impression of refractive index n_1, in contact with an immersion medium of refractive index n_2, it represents a limiting measurable value of $|n_2-n_1|\Delta S$. By decreasing $|n_2-n_1|$ the limiting value of ΔS, *i.e.* the departure from planeness of the under-surface of the impression and therefore of the surface under test, may be correspondingly increased. If the film on which the impression is taken is not plane on its upper surface, it may of course be cemented to an optically plane cover plate using a cement and glass of the same refractive index as the film. In this way the range of the instrument may be increased to enable undulations in the test surface of a depth of at least 20 μ to be measured. It will of course be clear that, whereas measurements of surface contour differences may normally be made to one tenth of a fringe (corresponding at the object to $\lambda/20$, or *ca* 1 μ in., under normal conditions), when $|n_2-n_1|$ is adjusted to $1/p$ the limit of precision of measurement is increased to $p\lambda/20$ (or $p\ \mu$ in.). An example of this application is shown in *Figure 5*, representing an interferogram of the tip of a Vickers diamond indenting tool used for hardness tests. The instrument described in this article is the subject of two current applications for Patent.

Thanks are due to the Director of the National Physical Laboratory and staff of the Light and Metrology Divisions, also to Mr C. Timms of the Mechanical Engineering Research Laboratory, for initial interest and constructive discussions; also to the staff of Hilger & Watts Ltd, especially Mr C. Godfrey, for valuable collaboration during the design and development of this instrument.

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METALLURGICAL ASPECTS OF MICROSCOPY

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The considerable progress which has been made in recent years in the microscopical examination of metals is reviewed. The most noteworthy achievements include the development of techniques involving phase contrast, polarized light and interferometry. Other highly specialized equipment has yielded useful information and will undoubtedly be developed further.

IN THE past two decades, the microscopical examination of metals advanced rapidly so that the metallurgical microscope has assumed greater importance. The increase in the number of types of illumination which can be used for examining opaque specimens has been matched by the improvements in the methods of preparing metal sections, notably in the greater control of the relative relief of constituents of different hardness and chemical nature. Diamond dust has been used[1] as an abrasive to preserve flatness in multi-phase specimens and satisfactory electrolytes have been found for the electro-polishing of a wide range of metals and alloys. In addition, there has been developed[2] the technique of attack-polishing in which the abrasive is suspended in acid solutions to reduce the amount of surface oxidation and mechanical flow during metallographic polishing.

The progress in metallurgical microscopy has aroused much interest and several authors have reviewed the subject within the past three years. In 1953, the Institution of Metallurgists held a refresher course in this field and five papers were given which included details of the uses of polarized light, phase contrast and interferometry in metallography; the papers have been published[3] and could profitably be consulted. At the American Society for Metals Seminar of 1952, G. L. KEHL[4] described progress in phase contrast and polarized light equipment and in reflecting objectives. Other papers emphasizing various aspects of microscopy have been given by E. C. W. PERRYMAN[5], B. W. MOTT[6] and R. C. GIFKINS[7].

Apart from the introduction of specialized components to give specific forms of illumination, general improvements have also been made in the optical train. For example, the introduction of binocular eyepieces has considerably reduced eye strain and the increased light intensity given by high pressure mercury vapour lamps has also been a great improvement. For a plain glass slip formerly used as a vertical reflector, the maximum light transmitted to the eyepiece is about 5 per cent of that supplied by the optical train. By coating the reflecting surface with a thin film of zinc sulphide or titanium oxide, the efficiency is increased to over 20 per cent and double image formation can be prevented by coating the second surface with magnesium fluoride. A further increase in efficiency of the vertical reflector can be obtained by the use of the Foster calcite prism[8], which was designed for the Bausch and Lomb metallograph and also served both as polarizer and analyser. Many modern metallurgical microscopes have a rotatable stage which is essential for polarized light work and useful with other forms of illumination.

PHASE CONTRAST

In 1935, F. ZERNICKE[9] published details of the phase contrast technique for transmitted illumination, in which components having different refractive indices could be revealed by virtue of the relative phase change which was converted into an intensity difference. About ten years after Zernicke's original work, the phase contrast technique was developed for opaque specimens for which the relative phase change was provided by differences in surface level, and the first paper describing the possible metallurgical applications was given by F. S. CUCKOW[10] in 1949, followed by several others[3,11,12]. The general uses of phase contrast in metallography include the examination of multi-phased alloys after light etching and of cleavage faces of single crystals, and the detection of precipitation at an early stage. The optimum range of difference in surface level is about 200 to 500 Å, although under favourable conditions some information can be obtained outside these limits.

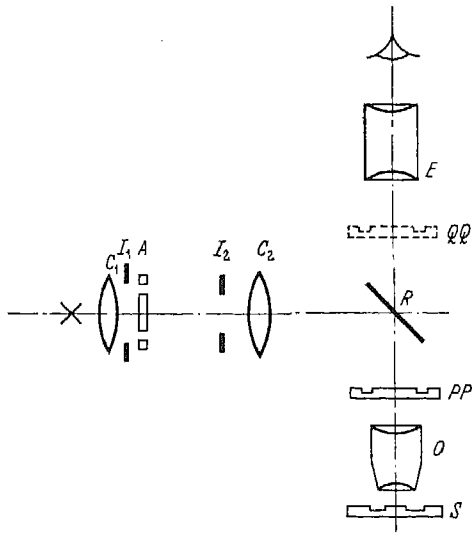


Figure 1. Basic arrangement for phase contrast with vertical illumination

The basic arrangement for phase contrast in the metallurgical microscope is shown in *Figure 1*. An annular aperture A placed behind the lamp iris I_1 gives a hollow cone of light with which the specimen S is illuminated. A phase plate of suitable size should strictly be positioned in the back focal plane of the objective O at position PP between the vertical reflector R and the objective, although some manufacturers prefer position QQ in front of the eyepiece E. The phase plate has an annulus, formed either by etching or deposition, such that the light it transmits is either advanced or retarded by a quarter of a wavelength relative to the light transmitted by the rest of the plate, resulting in positive or negative phase contrast respectively. With positive phase contrast, areas of the specimen which are proud of the matrix appear bright and pits dark. As an example of this, *Figure 2* shows a cleavage face of a single crystal containing twins, accommodation bands and other deformation characteristics. *Figure 2*a was taken under ordinary vertical illumination, *Figures 2*b and c with positive and negative phase contrast respectively, and *Figure 2*d using the opaque-stop technique described in the next section.

A more recent development of phase contrast[13] is due to M. FRANCON and G. NOMARSKI, and can be used to incorporate colour contrast in the image. The light reflected from the specimen passes through the two quartz plates cut at 45° to the optic axis and crossed, and interposed between two polarizing units. If a sensitive tint plate is inserted in the system, then colour differences can be obtained and the contrast can be changed by varying the inclination of the quartz plate system. The metallurgical applications of this type of apparatus have been described by G. NOMARSKI and A. R. WEILL[14], and include a study of dislocation growth spirals and of surface phenomena in deformed metals.

OBLIQUE AND OPAQUE-STOP ILLUMINATION

Surface irregularities can be revealed by either dark background or oblique illumination. For dark background work, the specimen is illuminated by a hollow cone obtained by stopping out the central rays which would strike the specimen at near-normal incidence; this results in surface irregularities, which diffract the light, appearing bright on a dark background. Alternatively, by suitably adjusting the vertical reflector, the specimen can be illuminated obliquely at full aperture. A. F. HALLIMOND[15] has described a stop-contrast device which renders differences in tilt of greater than 1° on the specimen surface as intensity variations in the image. A stop having opaque and clear annuli is placed at the back principal focus of the objective and transmits light reflected at normal incidence through the central clear annulus; light reflected from tilted surfaces will be either partially stopped by an opaque annulus or transmitted through the outer clear annulus.

All these methods are useful in promoting contrast in the image but their sensitivity is limited and accurate determination of the tilt angle cannot be made. By means of the opaque-stop technique of W. M. LOMER and P. L. PRATT[16], however, surface slopes of about 1′ at ×6 magnification and 1° at ×200 can be

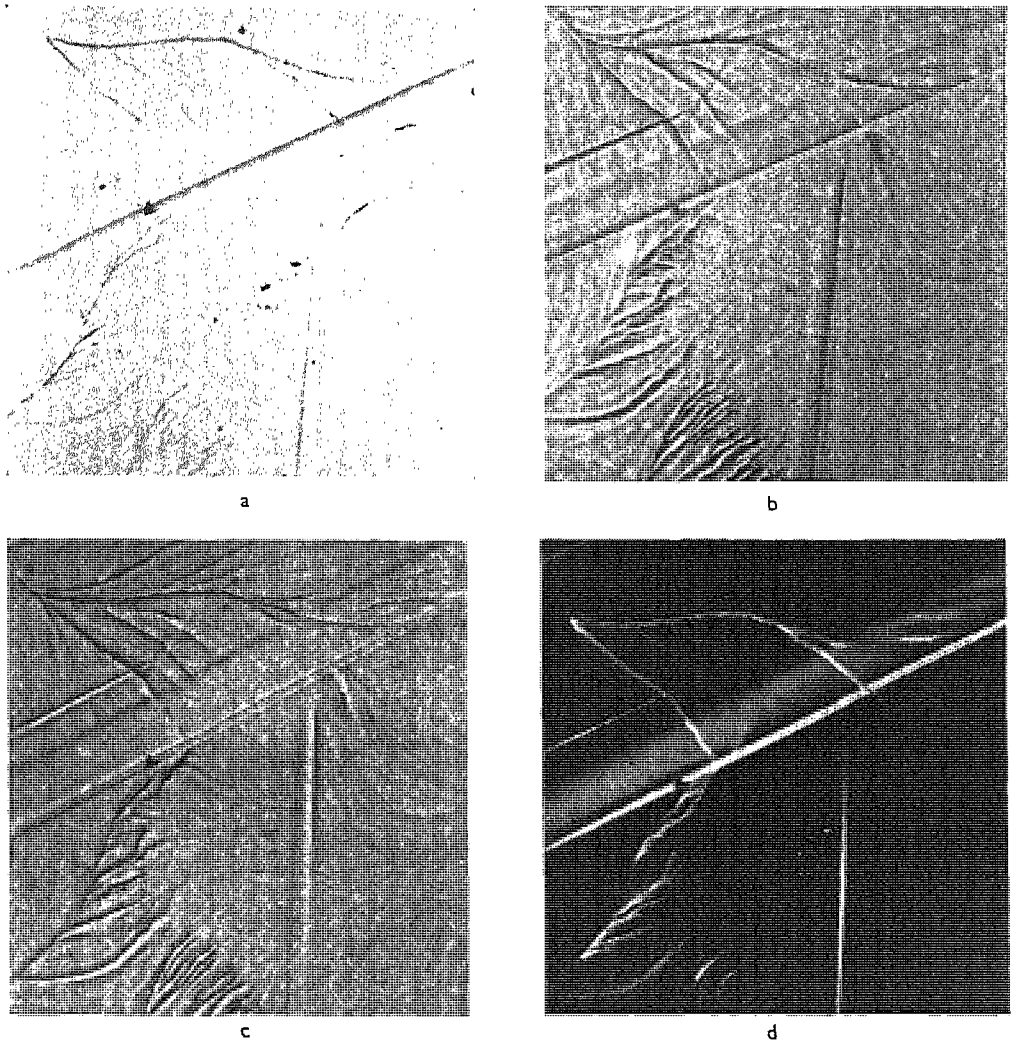


Figure 2. Cleaved zinc specimen: a *ordinary illumination;* b *positive phase contrast;* c *negative phase contrast;* d *opaque-stop illumination*

detected and measured. The principle of the method is to form the image with diffracted rays only and the apparatus is similar to that described for phase contrast work, except that an annular stop of the appropriate size is used instead of the phase plate. From measurements of the displacement of the image of the annular aperture due to reflection at an inclined face, the tilt angle of the face can be obtained. Alternatively, a straight-edge stop and point source may be employed so that the distance the stop has to traverse to eliminate light reflected from an incline can be used to estimate the inclination. Messrs Cooke, Troughton and Simms have constructed a slide which passes through a slot in the objective and incorporates a full aperture, positive and negative phase plates and an annular stop, so that a selection of four possible types of illumination may easily be made.

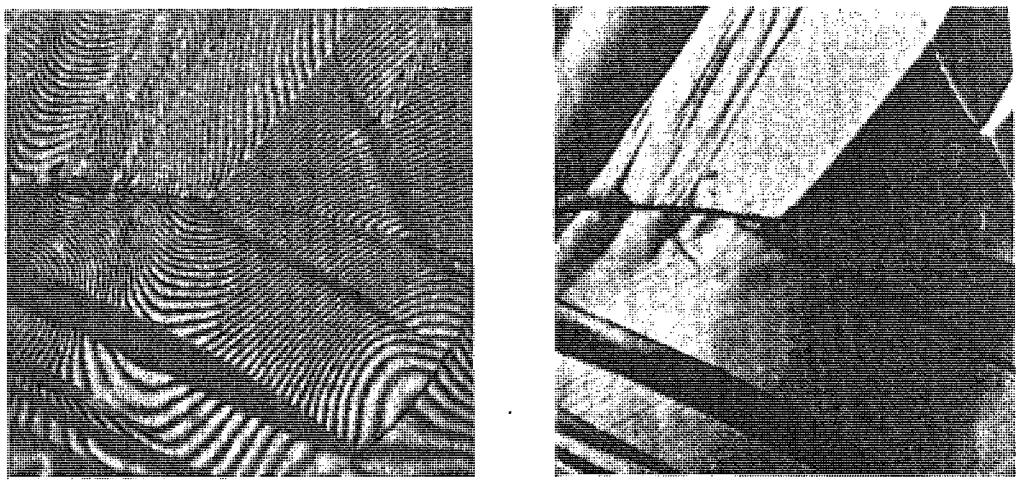
INTERFEROMETRY

Although surface topography has been investigated for many years by means of interference fringes, until comparatively recently the standard technique was to employ two beams only. In the last few years considerable improvement in sharpness and contrast has been obtained by the use of multiple beam

interferometry as developed by S. TOLANSKY. The fringes are produced by a wedge-shaped air gap formed between a specimen and a semi-silvered optically flat glass plate, and differences in level of down to about 25 Å are revealed by displacement of the fringes in the image. Ideally the incident beam should be monochromatic and parallel but a sufficient approximation to this may be obtained by stopping down the lamp iris and using low power objectives (focal length not less than 8 mm). Tolansky has given several examples of the use of multiple beam interferometry in his book[17] but the main metallurgical application has been to a study of deformation characteristics. Gifkins has shown[7] some beautiful photographs of interference fringes from deformed zinc specimens, an example of which is given in *Figure 3*a, while *Figure 3*b shows the same area under oblique illumination; the features revealed are slip, deformation bands and twinning. J. W. KELLY and R. C. GIFKINS[18] also employed interference techniques in a metallographic study of light transmitted by the objective so that part is focused on a silver spot and part on the specimen; interference occurs between the two beams when they are recombined at the objective and differences in level of about 30 Å can be revealed.

POLARIZED LIGHT

The use of polarized light in the microscopical examination of opaque specimens extends over more than half a century, but the number of applications has increased considerably in the past ten to fifteen years and, in addition, more attention has been paid to the efficiency of the apparatus than hitherto. Much of the impetus has been given by the usefulness of the technique in examining the new strategic metals, titanium, zirconium and uranium, and by the increase in the number of workers using the polarizing microscope for investigations on aluminium[22]. The whole field has been extensively reviewed[3,23,24] in recent years and can only be briefly outlined in the present paper.



a b

Figure 3. Deformed zinc: a *multiple beam interference;* b *oblique illumination (after Gifkins*[7]*)*

the cell formation and development in aluminium. S. TOLANSKY[19] has used his apparatus for investigating the distortions around diamond pyramid hardness impressions in materials having different recovery properties. For work at high magnifications, S. TOLANSKY[20] has suggested covering the specimen with a thin film of Canada balsam, but accurate control of the film thickness to give maximum definition of the fringes is not easy. Another apparatus for interferometry has been designed by J. DYSON[21] and is placed between the objective lens and the specimen. A half-silvered surface splits the

If correctly prepared, as-polished sections of anisotropic metals will ' respond ' to polarized light but isotropic metals need to be etched to invoke artificial anisotropy resulting from either non-cubic surface films or well defined pits which reflect the light at oblique incidence. In theory, the only requirements are that the incident light on the specimen be plane-polarized and that the reflected light be analysed by a polarizing unit with its vibration direction at right angles to that of the incident light. In practice, however, anomalous conditions may result[25] from strain in the objective

and non-uniform polarization over the field from the varying angles of incidence of the light reflected by the vertical reflector; these effects can be reduced to a minimum by suitable adjustment of various components and the use of a vertical reflector coated with zinc sulphide or titanium oxide. On most modern microscopes the polarizing units are made from polaroid sheet rather than Nicol prisms so that there is no restriction on the aperture.

One of the main uses of polarized light is to distinguish between areas of varying orientation which are revealed as differences of intensity under crossed polarizers. If the light reflected from the specimen has appreciable ellipticity, then a sensitive tint plate inserted in front of the analyser will result in each grain being identified by a characteristic colour which changes as the specimen is rotated. This technique can be used in the assessment of the degree of preferred orientation and in recrystallization investigations; papers illustrating the colour effects have been given by B. W. MOTT[6], and P. LACOMBE and M. MOUFLARD[26], and others[27].

Other uses of polarized light include distinguishing and identifying phases in multi-phased specimens. On stage rotation, a cubic phase remains extinguished whereas an anisotropic phase shows regular intensity changes; two non-cubic phases of sufficiently different anisotropy can often be distinguished when occurring together. A. M. PORTEVIN and R. CASTRO[28] and H. MORROGH[29] have discussed the identification of non-metallic inclusions in metals by using polarized light which will yield information on the shape, crystal symmetry, transmission colour and pleochroic properties. G. J. NEWTON and H. C. VACHER[30] have recently described attempts to correlate the orientation of the metal grain with measurement of the light intensity under crossed polarizers and with the form of the polarization figures in convergent light. For tin, a significant correlation was found between the extinction angle and the azimuth of the active axis, and the change in light intensity was approximately proportional to the fourth power of the sine of the angle between the optic axis and the surface normal. For aluminium and monel specimens, which have to be specially prepared to give optical anisotropy, the relationship between the reflected intensity and the orientation was not so evident.

USE OF ULTRAVIOLET LIGHT

The resolving power of an objective, *i.e.* the minimum distance between two lines which can be resolved, is directly proportional to the wavelength of the light and varies inversely as the numerical aperture. There is a definite limit to the numerical aperture of an objective and for visual work the minimum wavelength is limited by the sensitivity of the eye. As photographic plates are sensitive to ultraviolet radiation, this may be used to obtain not only an appreciable gain in resolution, but quite often increased contrast between constituents due to a more selective absorption of light of this wavelength.

Although F. F. LUCAS[31] first described the use of ultraviolet light in the examination of metals in 1934, E. W. TAYLOR[32,33] has recently given details of the technique employing modern equipment, and has illustrated the improvement in resolution by comparison photographs taken with visible and ultraviolet radiation respectively. Radiation of wavelength in the range 2500 to 2800 Å can be provided by a cadmium, mercury, magnesium or hydrogen arc, and all optical components which transmit the light must be made of quartz because of the high absorption of ultraviolet radiation by glass. The objective may be of the reflecting type, in which case the specimen can be focused in white light and photographed in ultraviolet; alternatively a fluorite or quartz monochromat lens may be used, when focusing is accomplished by means of a fluorescent screen placed in the focal plane of a special viewing eyepiece.

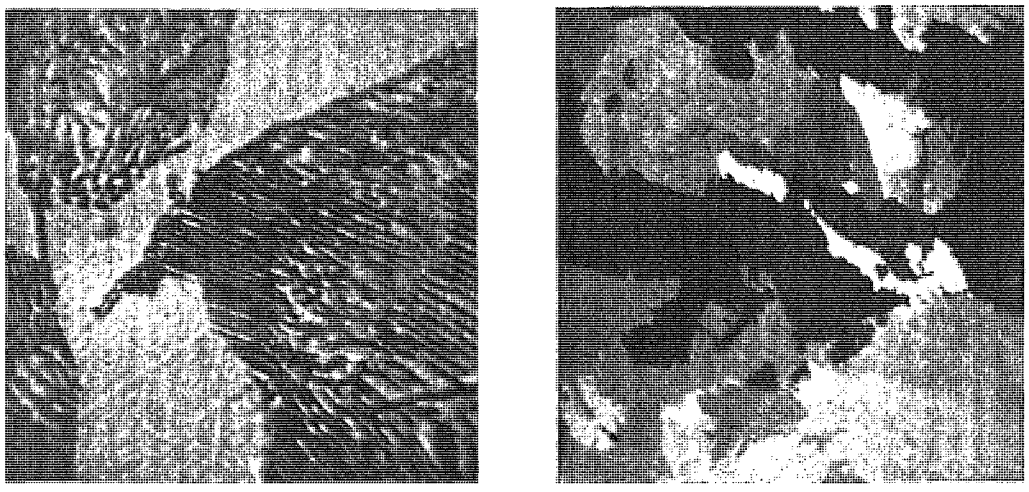
Increased resolution can also be obtained by means of the electron microscope and the work to date in the metallurgical field was adequately reviewed at a symposium[34] held at the Royal Institution in 1950. It has also been claimed that the resolution of the flying spot microscope is greater than that of the optical microscope, but no details have been published so far of its successful application to the examination of opaque specimens.

EXAMINATION OF HOT METAL SURFACES

Until recently, the microscope was used for the examination of metals at room temperature only, so that it was limited to structures which were either stable under these conditions or could be obtained in the metastable state by quenching from higher temperatures. Several advantages may be gained by studying structural changes such as recrystallization, grain growth, precipitation and phase transformations at the temperatures at which they occur, and several workers have operated equipment with which continuous microscopical examination may be made at temperatures up to about 1000°C. Since many metals oxidize rapidly in air to form films which would soon obscure the structure, it is usually necessary to contain the specimen in a chamber which can be evacuated or filled with an inert gas. Observation is made through an optically worked silica window so that the working distance of the

objective must be sufficient to clear the window. For this reason, a reflecting objective is highly suitable for this type of work, although a 16 mm transmission objective can be used if only low magnifications are required. As an alternative, the working distance can be improved by employing the attachment designed by J. DYSON[35] which enables a 4 mm objective to be used at a distance of about 13 mm from the specimen.

shielding equivalent to that afforded by about 6 in. of lead or 30 in. of concrete may be necessary for the protection of personnel handling quite a small specimen. It may be required metallographically to polish such a specimen and to examine its structure under the microscope. In the United States, the standard practice has been to reflect the light from the microscope through the shielding walls so that direct radiation from the specimen is impossible.



a b

Figure 4. Photographs from television screen in remote metallographic equipment: a *pearlite in mild steel;* b *zirconium under polarized light*

P. J. E. FORSYTHE[3] has reviewed the early work on the examination of hot surfaces and this includes a study of graphite formation on the surface of hypereutectoid steels by G. C. SMITH and M. J. OLNEY[36], the recrystallization of copper by J. R. MARÉCHAL and M. DOUCET[37], and the structural changes which occur in pure zinc on heating both with and without the application of external stress[38].

Using polarized light to reveal the grain structure at elevated temperature, recrystallization studies of zinc were made by G. BRINSON and A. T. W. MOORE[39], and of uranium by B. W. MOTT and S. D. FORD[40]. Examinations under polarized light are generally limited to relatively low temperatures, however, because of the high oxidation rate in the highest readily obtainable vacua *i.e.* $\sim 10^{-6}$ mm pressure of mercury.

MISCELLANEOUS DEVELOPMENTS

One of the many problems associated with atomic energy is the examination of radioactive metals after neutron irradiation in a reactor. Depending on the nature of the metal and the total neutron flux,

There are two objections to this system if polarized light examination is required; the light intensity, which is already low in the crossed-polarizer condition, may be further reduced by repeated reflection and in addition the state of polarization may be seriously affected. To overcome these difficulties, equipment has been built at Harwell in which the image is projected on to a television camera and finally displayed on a 15 in. cathode tube by means of a closed circuit. The image obtained has good definition at magnifications of up to $\times 10{,}000$ and above. This is illustrated in *Figures 4*a and b which respectively show pearlite in a mild steel and the grain structure in zirconium under polarized light; both photographs were taken with a studio camera direct from the screen.

An apparatus which can often be fitted to a metallurgical microscope is one for making diamond pyramid hardness tests at low loads *i.e.* in the range 1 to 200 gm. Since the diagonal of the impressions obtained may be down to about 1 μ, it is essential that good quality microscope equipment be available for their measurement and many manufacturers have

designed hardness testers for use with their metallographs. The main uses of low load testing include the hardness measurement of the phases present in an alloy (this may give valuable assistance in their identification), and the determination of hardness gradients such as those in diffusion couples, cored structures, surface hardened specimens and small fabricated components.

Other recent developments include a flat-field microscope objective of 6 mm focal length and 0·8 numerical aperture as described by B. O. PAYNE[41]. This gives a field too large to be accommodated by an ordinary low power eyepiece and must therefore be used in conjunction with a special ×8 eyepiece or a higher power compensating eyepiece. In the photography of metallic powders, it is often required to show both the shape and the surface topography and this is best achieved by a combination of lighting. B. W. MOTT and S. D. FORD[42] have described a technique of suspending the powder in a suitable mixture of glycerol and water, and illuminating simultaneously by both vertical lighting and light transmitted through the glass slide holding the suspension. A microscope on which this double lighting system can be readily achieved has been recently marketed by the Reichert Optical Company, Vienna, and described in its trade literature.

GENERAL REMARKS

Undoubtedly the value of the metallurgical microscope has been considerably increased in recent years by the introduction of new techniques and the improvements in existing equipment. Many observations made with a modern microscope need careful interpretation but this is often facilitated if the metallographer is prepared to use more than one method both for preparation and examination. It is essential to obtain as much preliminary information as possible under ordinary vertical illumination and then to select the most useful combination of specialized forms of lighting to further the knowledge gained.

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PHASE CONTRAST MICROSCOPY

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The phase contrast microscope is a development of the last twenty years. It has a number of advantages: it is easy to operate and can be used for studying living cells and transparent objects. In this article the author gives an explanation of the theory of phase contrast and its application to the measurements of the solid content of cells, particularly normal and abnormal blood cells.

THERE CAN BE little doubt that from the point of view of the biologist phase contrast is by far the most valuable method of microscopy yet invented. This fact has been recognized by the award of the 1953 Nobel Prize for Physics to its discoverer, Professor FRITS ZERNIKE of Groningen, Holland. Curiously enough the first applications of the method were not to microscopy, but to the study of diffraction gratings[1] and astronomical telescope objectives[2]. F. ZERNIKE[3] soon realized the important possibilities of phase contrast microscopy, as did also C. R. BURCH[4], and attempted to interest microscopists and manufacturers in the subject. It is amusing now to look back and reflect on the very conservative and unenthusiastic reception which was given to these early efforts. Indeed, one microscope firm went so far as to suggest that, had the method been any good, they would have discovered it themselves.

However, progress became more rapid after the last war, and phase contrast equipment is now produced by almost all leading manufacturers throughout the world. It has become a routine and essential method for all biologists interested in studying the structure and behaviour of living cells. Only a very brief account can be given here; several monographs and reviews exist and should be consulted for further details[5–9].

THEORY

It is difficult to give a rigorous explanation of phase contrast without elaborate mathematics and indeed the full theory as applied to the actual conditions used in practice has not been worked out. Perhaps the simplest way to approach the matter is to consider what happens when light passes through (*1*) a partially absorbing object and (*2*) a completely transparent object in the microscope. In *Figure 1*a let the curve *A* represent a light wave falling on the object. The intensity of the light will be proportional to the square of the amplitude of this wave. After passing through a partially absorbing object, the intensity is reduced, so that the emerging wave is represented by *B*, which is similar to *A*, except that its amplitude is lower. However, the emerging wave can be represented in another way; it can be regarded as the sum of the original wave and of another wave, shown dotted in *Figure 1*b. It is easily seen that if the two waves in *Figure 1*b are added together (taking upward displacements as positive and downward displacements as negative) they will give wave *B*.

This method of representation is not a mere mathematical fiction, for both the waves shown in *Figure 1*b have a real physical existence. The wave *A* is the incident wave; the dotted wave owes its existence to the presence of an object. It is in fact the wave which has been produced by diffraction at the object. The final image is obtained by the interaction or interference of these two waves.

Now consider a perfectly transparent object. Since no energy is lost, the amplitude of the wave is unaltered by passing through the object. However, due to the refractile properties of the object the transmitted wave will be either advanced or retarded in phase relative to the incident one. In *Figure 2*a the transmitted wave *B* is shown as retarded relative to *A*. As in the case of an absorbing object the transmitted wave *B* can be represented as the sum of the incident wave *A* and a diffracted wave, which is shown as a broken line in *Figure 2*b. An important difference between this wave and the corresponding one in *Figure 1*b is at once obvious. Whereas in the case of an absorbing object the diffracted wave is exactly half a wavelength (180°) out of phase with the

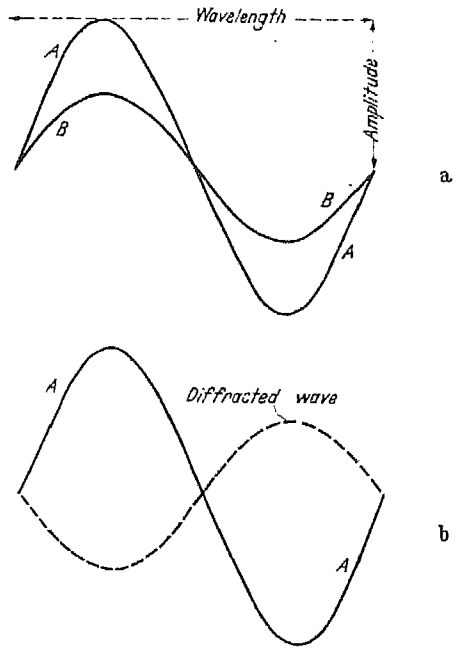

Figure 1. Image formation in the case of a partially absorbing object: A represents the incident wave, B the transmitted wave, which is reduced in amplitude; B can also be represented as the sum of A and a diffracted wave shown in b; the diffracted wave is 180° out of phase with the incident wave

incident wave, so that the crest of one corresponds with the trough of the other, the wave diffracted by a transparent object is out of phase with the incident wave by a variable amount which depends on the refractile properties of the object (*i.e.* on the product of the thickness and the difference in refractive index between the object and surrounding medium). For the weakly refractile objects frequently encountered in microscopy this phase difference is roughly one quarter of a wavelength as shown in *Figure* 2b.

We now come to the essential feature of phase contrast; by means of an optical device the phase difference between the diffracted and incident wave is increased from about 90° to about 180° so that the two waves can cancel each other out as far as possible. This is shown in *Figure* 2c in which the phase of the diffracted wave has been altered so that its crests correspond in position with the troughs of the incident wave. The sum of these two waves is the new resultant wave and on comparing *Figures* 2c and 1a it will be seen that it corresponds to the transmitted wave in the case of an absorbing object. To the eye and the photographic plate an otherwise transparent object would appear as if it were a partially absorbing one. This is the reason why phase contrast is so important to biologists. Most living cells are virtually completely transparent so that very little can be seen by conventional microscopy. Various parts of the cell differ, however, in refractive index and thickness, and these differences are transformed by phase contrast into variations in intensity so that the transparent cell appears to be stained in shades of grey. This result is obtained without any sacrifice in resolving power or image quality.

Before considering how this is achieved brief reference will be made to the vector method of representation which is most helpful in dealing with many problems in interpreting phase contrast images. This method was first used qualitatively by Zernike[3], but has recently been extended and developed quantitatively[10]. The results of this simple theory are essentially identical with those obtained by far more elaborate mathematical

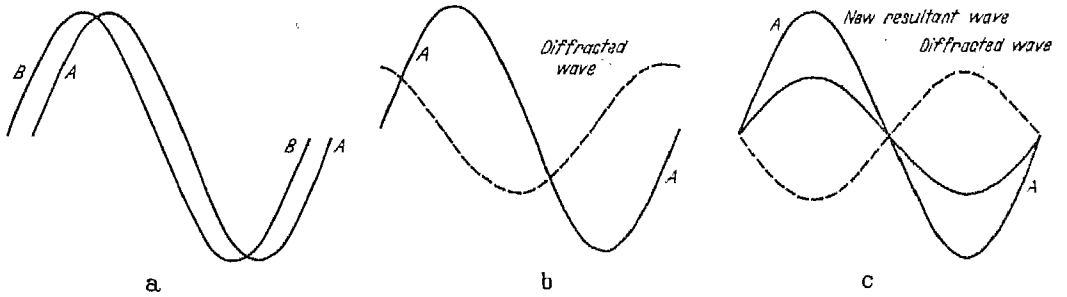

Figure 2. Image formation in the case of a transparent object: the transmitted wave B is unaffected in amplitude, but slightly delayed in phase; B can be represented as the sum of A and the diffracted wave shown in b; these two waves are approximately 90° out of phase—thus, if a further phase difference of about 90° is added, the two waves will be 180° out of phase and will add up to give the new resultant wave, which is reduced in amplitude

analysis. In this method light waves are represented in phase and amplitude by vectors. In *Figure 3* the incident wave is represented by O_1A. Its amplitude is O_1A and its intensity O_1A^2. After passage through a transparent object detail, the emergent wave will be unaltered in amplitude, but changed in phase. It will thus be represented by a vector such as O_1p, having the same length as O_1A, but making a phase angle $pO_1A=\phi$ with it. Thus all transparent details will be represented by such vectors differing only in the value of ϕ so that their ends fall on the circumference of the circle centre O_1, radius O_1A. This may be called the object circle. Now, by the usual laws of vector addition O_1p is the sum of O_1A and Ap: O_1A represents the incident wave and Ap represents a new wave ' created ' by the object,

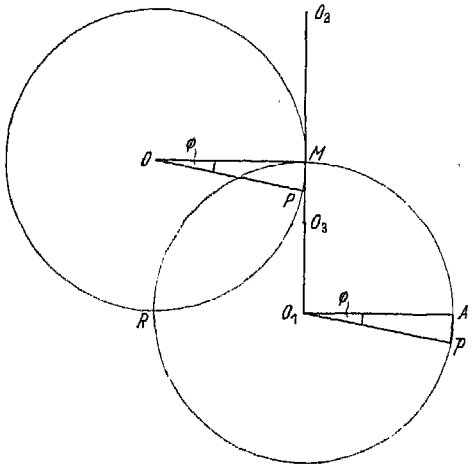

Figure 3. Vector representation of phase contrast

in other words the diffracted wave. It will be seen that when ϕ is small these two vectors are roughly 90° out of phase. As already explained the purpose is to make them 180° out of phase. This can be done in the vector diagram by rotating O_1A through 90° to O_1M, thus altering the phase of the incident wave without affecting the diffracted wave. These two waves now recombine to form the image. This is equivalent to adding the vectors O_1M and Ap ($=MP$). The resultant is O_1P. Since MP is equal and parallel to Ap it follows from the geometry of the figure that every point such as p on the object circle is transferred to a point such as P.

For different values of ϕ, P traces out a circle centre O, radius $OM=O_1A$. This new circle is called the image circle. The object of this procedure is that, instead of the intensity of all transparent details being equal ($=O_1p^2$), the intensity O_1P^2 varies as a function of ϕ. In the case illustrated a simple calculation shows that the intensity is given by

$$I=O_1P^2=3-2\sin\phi-2\cos\phi$$

For very small values of ϕ this approximates to $I=1-2\phi$, that is, the intensity varies approximately linearly with the phase change. It is obvious, however, that this linear relationship does not hold when ϕ is large. When ϕ is zero, the intensity is given by O_1M^2. This is therefore the background intensity obtained in a region of the field of view free from object detail. As ϕ increases, the intensity decreases, passes through a minimum and then rises again. For the case illustrated when $\phi=90°$ (corresponding to an image point R), the intensity O_1R^2 is once again equal to the background intensity O_1M^2 so that contrast will be zero. When ϕ exceeds 90°, the image intensity will exceed that of the background so that the image will appear with reversed (bright) contrast.

Having seen how the image circle can be derived, there is no need to consider the object circle further. We can simply draw the image circle centre O, radius OM and do all our calculations by changing the origin of the vectors from O to a new point. Thus, if the phase of the direct light is advanced by 90° as in the above example, OM must be rotated through 90° about M. The new origin will then be O_1. We need not, of course, make this phase change exactly 90°—indeed for some purposes other values may be preferable—but the 90° condition is most often used in practice because it can be shown that it gives the greatest sensitivity for weakly refractile details (*i.e.* when ϕ is small). In some cases it may be useful to retard the phase of the incident wave instead of advancing it. A 90° retardation would bring the new origin to O_2. In this case the image intensity O_2P^2 will generally exceed the background intensity O_2M^2 and refractile details will appear bright instead of dark. This is known as negative phase contrast—the first type in which the direct light is advanced in phase being known as positive phase contrast.

Another important device frequently used in practice is to absorb part of the incident wave as well as alter its phase after it has passed through the object. On the vector diagram this is equivalent to shortening O_1M so that the new origin lies at, say, O_3. This has the effect of increasing the contrast when ϕ is very small. It will be seen from *Figure 3* that under these conditions O_1P is very nearly equal to O_1M whereas the relative difference between O_3P and O_3M is much greater.

These are only a few examples of how the method may be used, and the behaviour of any given system

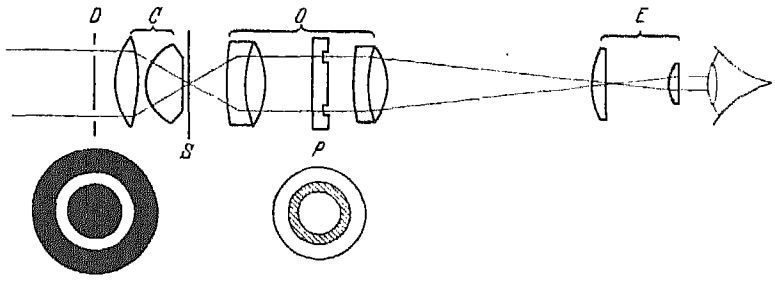

Figure 4. Optical system of phase contrast microscopy

can be visualized from the diagram without going into detailed calculations. Exactly the same type of representation can be used in the case of interference contrast and indeed from the vector theory point of view the two methods are identical, though important differences exist in practice. Phase contrast is in fact a special type of interference contrast.

PRACTICAL REALIZATION

In order to obtain phase contrast we must be able to separate the incident or direct wave from the diffracted wave. In an ordinary microscope light is focused on the specimen slide S by the condenser lenses C (*Figure 4*). In the absence of a specimen there will be no diffracted waves so that the light passing through the optical system will be the direct or incident light alone. If the diaphragm D is placed at the focal plane of the condenser an image of it will be produced at the rear focal plane of the objective O and if the eyepiece E is removed an image of D will be seen at the back of the objective.

If now a specimen is introduced, some light will be diffracted by it. This light is spread out to fill the whole of the back lens of the objective and will overlap the direct light. If the diaphragm D is in the form of a circular hole, as is normally used in conventional illumination, the degree of overlap will be so great as to make separation of the two types of light impossible. If, however, we make D conform to some special shape such as a slit, or an annulus as illustrated, the direct light will form an image of this annulus, but the diffracted light will be evenly spread over the whole of the back lens of the objective. Although there will be some degree of overlap between these two components, this will be restricted to the comparatively small area occupied by the image of the annulus.

Having achieved this degree of separation, it is now necessary to alter the phase (and usually the amplitude) of one wave relative to the other. This is done by inserting a phase plate at the rear focal plane of the objective. This is shown at P in *Figure 4*. The phase plate is represented as a transparent disk containing an annular groove. The latter is made to coincide exactly with the image of D formed at this plane. In this way all the direct light is made to pass through the groove whereas most of the diffracted light will pass outside the groove. The depth of the groove is adjusted to give the desired phase difference between the two sets of waves. Reduction of intensity of the direct light can be achieved by depositing a thin metal film on the groove. There are of course many different ways of making phase plates, but the one illustrated indicates the general principles. The phase plates are usually incorporated in the objectives, and it is sometimes necessary to position them between the lenses. Such phase contrast objectives can, however, be used with conventional illumination at the cost of a slight loss of image quality and contrast.

The diaphragm D has to be matched to the phase plate in each particular objective, and it is usual to arrange a number of annular diaphragms of different sizes on a rotating turret attached to the microscope condenser. There are no other special requirements and in practice the apparatus is very simple and convenient to use. This feature makes it particularly valuable for routine work, in contrast to the rather elaborate and occasionally temperamental interference microscopes.

Many other types of phase contrast microscopes have been described, but few of them are as satisfactory as the simple basic type. One disadvantage of the latter is that, as the phase plates are built into the objectives, a special objective has to be bought every time one requires to change the phase plate. In practice it is not often necessary to do this and a reasonable compromise can be achieved for most types of work by using only one type of phase plate. The additional flexibility conferred by being able to change the phase plate is nevertheless very useful, and several devices for enabling any objective to be used with interchangeable phase plates have been described.

One of the best of these devices[11] is illustrated in *Figure 5*. The essential problem is to place a phase

plate at some accessible position. The rear focal plane of the objective at which the phase plate is normally situated is often inaccessible, but it is always possible to form an image of the rear focal plane and make the phase plate coincide with this image. An aluminized reflecting prism *R* is placed as shown above the objective *O*. This deflects the image beam on to a concave mirror *M* which is tilted very slightly so that the beam returns to the upper face of the reflecting prism and thence along the axis of the microscope. The primary image is formed near *M* and is then re-imaged by an auxiliary lens *L*, to be viewed through a normal eyepiece. The rear focal plane of the objective is imaged by *M* at *P*, at which level a phase plate is inserted. The condenser *C* is provided with the usual annular

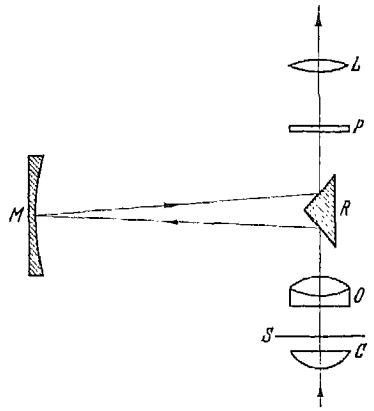

Figure 5. Diagram of optical system which enables interchangeable phase plates to be used with any objective

diaphragm. In this way an ordinary objective can be used and phase plates can be exchanged at will. The instrument can also be used with special quartz phase plates and polarized light. This enables the characteristics of the phase plate to be varied continuously, and positive or negative phase contrast can be obtained simply by rotating a polarizer. More detailed descriptions of the many different types of phase contrast systems invented have been published elsewhere[5-9].

Practical defects—The simple theoretical discussions given above do not take into account certain troublesome features which become apparent in practical phase contrast systems. It has been assumed that the direct and diffracted waves can be completely separated; in practice, however, there is always some degree of overlap due to the finite width of the phase plate annulus. This results in dark object details being surrounded by a bright halo and *vice versa*. Phase contrast cannot create or destroy light energy; it can only re-distribute it. Thus, if one part of the image field appears darker than the average level of illumination, another part will appear brighter. A related effect may be called the 'zone of action'. By this is meant the heightening of the phase contrast effect near edges and discontinuities, and its progressive falling off towards the interior of a uniform object. This is not necessarily a disadvantage for observational work, but makes certain quantitative measurements difficult. These defects are due to the fact that phase contrast is an 'imperfect' form of interference contrast, in which the two interfering beams cannot be completely separated.

APPLICATIONS

Phase contrast is now being used as a routine method for studying living cells and indeed transparent objects of any sort. It is impossible to mention all the types of investigations which have been carried out with it and fuller details of which have been published elsewhere[5-9]. In general the method has been most valuable for studying the growth and division of cells and for observing their reactions to such things as drugs, radiations and other physical and chemical agents. Many fine cinematograph films of cellular division and activity have been taken and these processes have been followed in far greater detail than was possible before[12]. In fact the use of phase contrast for purely observational purposes calls for little comment nowadays since it has virtually superseded most other methods so far as living cells are concerned. Its more recent use as a quantitative method is perhaps less familiar and requires more detailed discussion.

CELL REFRACTOMETRY[13-15]

Method—Since phase contrast works by converting differences in the product of refractive index and thickness into differences in light intensity, it is essentially a method of micro-refractometry. Unfortunately it cannot be used for measuring refractive index directly, but it can be used as a very sensitive null method. Thus, if an object is immersed successively in a series of media of different refractive index and then observed by positive phase contrast, it will generally appear dark when its own refractive index exceeds that of the medium, bright in a medium of higher refractive index, and invisible in one having the same refractive index as its own. This principle of matching refractive indices is of course well known and was used in crystallography and mineralogy long before the invention of phase contrast. The latter is so much more sensitive than older methods of illumination that it has enabled the

accuracy of matching to be very greatly increased so that refractive indices can usually be determined with an accuracy about ten times greater than previously.

Nevertheless, there did not seem much hope of applying the immersion method to living cells because most of the substances used in mineralogy are oils or organic solvents, and harmful to cells. Salts or other substances of low molecular weight are also found to be unsuitable because of the very high osmotic pressures which they exert in solution. Most living cells are very sensitive to osmotic changes and can only survive within a comparatively narrow range of pressures. A few years ago an accidental observation in the author's laboratory led to a solution of the problem and also showed how the results could be interpreted in a most useful manner. As already mentioned, living cells viewed by phase contrast usually appear dark because they have a slightly higher refractive index than the dilute salt media in which they are generally examined. One day, however, it was noticed that the free-living cells found in earthworms' blood appeared bright instead of dark. This was most mysterious as it could only mean that these cells had a lower refractive index than that of the medium and similar observations had not been made before. It was soon realized, however, that the cells were not immersed in the usual dilute salt solution, but in earthworms' blood which contains a high concentration of the protein respiratory pigment, haemoglobin, in solution and not locked up within cells, as in higher animals. In order to determine the refractive index of the cell one only had to dilute the blood until the peripheral part of the cell (*i.e.* the cytoplasm) became invisible.

This observation at once suggested a new line of approach. The reason why cells can survive in concentrated haemoglobin solution is that the latter has a high molecular weight (about 70,000) and thus exerts an almost negligible osmotic effect as compared with salts, which are present in much smaller amounts. Haemoglobin itself is not a very convenient substance to use because it is deeply coloured, rather unstable and not readily available commercially. Other proteins and substances of high molecular weight were tried, and it was found that bovine plasma albumin, which can be bought as a dry powder, had the necessary properties. It is soluble in water (up to about 55 per cent), practically colourless and exerts a very low osmotic pressure, so that salts can be added to make a 'physiological' medium in which cells can survive. The adjustment of osmotic pressure is very important since, if the cells swell or shrink, their refractive index will change. Particular attention has therefore been paid to checking the osmotic effect by measurements of freezing point depression and by comparing the diameters of spherical cells in the medium and in standard salt solutions. Other proteins such as egg albumin and plasma globulin have also been used and even acacia gum is suitable for some purposes. Nevertheless bovine plasma albumin remains the most generally useful and convenient material.

Interpretation—The main constituents of living cells are proteins. If a solution of these proteins could be made and used as the immersion medium, we might reasonably expect that, when the refractive index of the medium matched that of the cell, the concentration of protein in each would be the same. Might this also be true for different proteins? Fortunately this is in fact the case. It has been shown experimentally that for many substances in aqueous solution there is a linear relationship between refractive index and concentration *i.e.*

$$n = n_S + \alpha C$$

where n is the refractive index of the solution, n_S that of the solvent (either water or a dilute salt solution), C is the concentration expressed in gm/100 ml of solution and α is a constant known as the specific refraction increment. This constant has been determined for many different proteins and does not usually differ from a mean value of 0·00185 by more than $\pm$ 5 per cent. The refractive index measurement can therefore be converted into protein concentration with good accuracy. There are, of course, other substances beside proteins in living cells, but usually these only occur in relatively small amounts and in any case they have values of α not greatly different from those for proteins. We can reasonably take the mean refraction increment for all the protoplasmic solids as 0·0018 and in this way convert our readings into concentration of total solids rather than of proteins. Knowing the value for solid concentration, we can readily find the water concentration. This is not simply $100-C$ because one gram of solid does not necessarily occupy 1 ml in solution: $100-0{\cdot}75C$ is a better estimate.

To summarize, therefore, a simple measurement under the microscope is capable of giving the solid and water concentration of living cells with quite a high degree of accuracy. The usual method of determining these quantities requires large amounts of tissue and involves tedious drying and weighing. It is doubtful if the latter could be applied to single cells; it can certainly tell us nothing about living cells.

Results—Many measurements have been made on a wide variety of cells including bacteria, fungi, protozoa, blood cells, tumour cells, and cells grown in tissue culture. Accurate measurements can only be made on those parts of the cell which come into actual contact with the immersion medium. Cell inclusions such as nuclei cannot be measured, though it is possible to say whether they have a higher or lower solid content than the cytoplasm. In most animal cells the solid content of the cytoplasm lies between 10 and 20 per cent. Red blood cells, however, contain much more—about 35 per cent. Bacteria are relatively solid structures with between 25 and 45 per cent. Some structures, such as fungal spores, contain more than 55 per cent of solids and their exact content cannot be measured by this technique. In general the cytoplasmic concentration of resting cells is very uniform and no marked gradients exist in different parts of the same cell.

This does not necessarily apply to cells in motion or to dividing cells. It is interesting to observe an amoeba immersed in, say, an 8 per cent solution of protein. The resting pseudopodia usually contain about 10 per cent of solids and appear dark. When the creature moves, however, and a pseudopodium is protruded, the latter appears bright, and contrasts remarkably with the dark resting regions. The solid concentration in an active pseudopodium may fall to only about 2 per cent. This indicates that during activity either water enters the active region or, perhaps less likely, solids are transported from it to the inactive parts.

A less dramatic fall in refractive index occurs during cell division. The meiotic divisions in the germ cells of insects have been studied in some detail. Grasshopper cells are particularly suitable and will grow and divide in the protein medium for periods of more than three days. In a resting primary spermatocyte the cytoplasm contains about 13·5 per cent of solids. There is a progressive fall during the early stages of division and a minimum of about 10·8 per cent is reached in early metaphase. This is followed by a gradual rise up to about 11·7 per cent as the two daughter cells (secondary spermatocytes) are formed. Further division of the daughter cells is also accompanied by similar changes in solid concentration. These differences are not great, but are quite significant, particularly when viewed in relation to the very considerable changes in cytoplasmic volume which occur during division. Measurements on several thousand cells have shown that the cytoplasmic volume may undergo a twofold or even threefold increase during division. If this were due to an imbibition of water alone, the solid concentration should decrease by at least 50 per cent. In fact it only decreases by about 20 per cent. It is evident therefore that the solid concentration is being maintained by the production of a very large amount of new material in the cytoplasm, either by active synthesis or possibly by transference from the nucleus to cytoplasm.

An extensive investigation has been carried out on the solid concentration of normal and abnormal blood cells. An important feature here is that, since about 97 per cent of the solids in red cells is haemoglobin, the method gives a direct measure of the haemoglobin concentration in individual cells. Previously this important quantity could only be determined by indirect methods, which gave an average value for millions of cells. The new method allows a distribution curve to be obtained, showing the proportion of cells containing a given haemoglobin concentration. This sounds very tedious, but in fact the method is ideally adapted for statistical work and does not require detailed measurements on large numbers of individual cells. A very small drop of blood is put into a larger drop of a given protein solution; the cells which have a higher refractive index than the solution appear dark, and those with a lower index bright. The relative proportion of these categories is estimated and the procedure repeated with several protein solutions of different concentration. This method can of course be used with any type of cell population and not merely for red cells alone.

The range of concentration found in red cells from normal persons is 27 to 35 per cent, with a mode at 30 to 31 per cent. Considerable deviation from the normal pattern and values are found in many blood diseases. In iron deficiency anaemia all the values are much lower with a mode between 23 and 29 per cent, and a slightly wider range. The upper limits of the range rarely exceed the lower limits of the normal range, however. Perhaps the most interesting findings have been in certain haemolytic anaemias, in which, although the total haemoglobin in the blood is reduced, the haemoglobin concentration in the individual cells is actually considerably raised. The mean values in many haemolytic anaemias lie between 33 and 37 per cent and in many cases a high proportion of the cells contain over 40 per cent or even 45 per cent of solids whereas values above 33 per cent are rare in normal cases. This finding has been so consistent as to be virtually diagnostic. A number of these cases have been followed after treatment by removal of the spleen. In the acquired type of haemolytic anaemia the blood picture returned almost completely to normal, but in the congenital

type, although the values became lower, they were still above normal.

Further aspects—Another feature of the method is its value for purely observational work on certain types of cells. In phase contrast microscopy of non-living specimens it is often very useful to mount the latter in media of different refractive indices because in that way the relative refractile properties of different parts of the specimen can be varied and structural details differentiated and emphasized. The use of a protein medium enables the same thing to be done with living cells and by means of a range of different concentrations first one part of the cell and then another can be made paler, invisible or bright. This can be regarded as a method of 'optical dissection'. Immersion in protein media is particularly useful in the case of certain spherical cells. When observed in saline media, the refractive index difference between the cell and the medium may be so great as to make the cell behave like a small lens. This effect disturbs the operation of the phase contrast system and most of the internal detail of the cell may be obscured by a bright halo. The lens-like action can be reduced by raising the refractive index of the immersion medium and, when this is done, the internal details of spherical cells can be clearly seen. Without this device it would be necessary to compress the cells to view their internal structure, but this inevitably causes damage.

The fact that living cells can be made to appear bright in a protein medium depends on their ability to keep out protein from the cell interior. If this permeability barrier is damaged, however, protein will leak into the cell and it will be impossible to bring about reversal of contrast. This suggests a method of distinguishing between dead and living cells and of studying the action of various agents on cell permeability. It has been found that treatment by many substances, particularly formaldehyde, osmium tetroxide, mercuric chloride *etc*, which are used for fixing and killing cells and also many fat solvents such as alcohol, ether and chloroform will cause a rapid loss of the permeability barrier. Simple air-drying, even for a very short time, will do the same, particularly in the case of red blood cells. The latter also begin to lose their impermeability to protein if kept between a slide and cover slip for more than 24 hours, but other cells appear to be less susceptible and, though they undergo considerable swelling, are still capable of excluding protein.

Recently a remarkable exception to the general rule that cells cannot be reversed in contrast in protein after drastic chemical treatment has been observed in the case of chromosomes. Even after thorough drying or treatment with formaldehyde or alcohol the chromosomes can be made to appear bright by immersion in fairly concentrated protein solutions. The refractive index in the protein medium is generally about 1·40. This seems curious because it is well known that the refractive index of a dried protein film or fibre is usually about 1·54.

In fact, if one immerses these cells in non-aqueous media, such as are used in mineralogy, the chromosomes appear dark in media of much higher refractive index than 1·40 and only disappear at a refractive index of about 1·54. In other words, the chromosomes seem to have a different refractive index according to whether this is measured in an aqueous or non-aqueous medium. Further investigations have suggested that the nucleoprotein

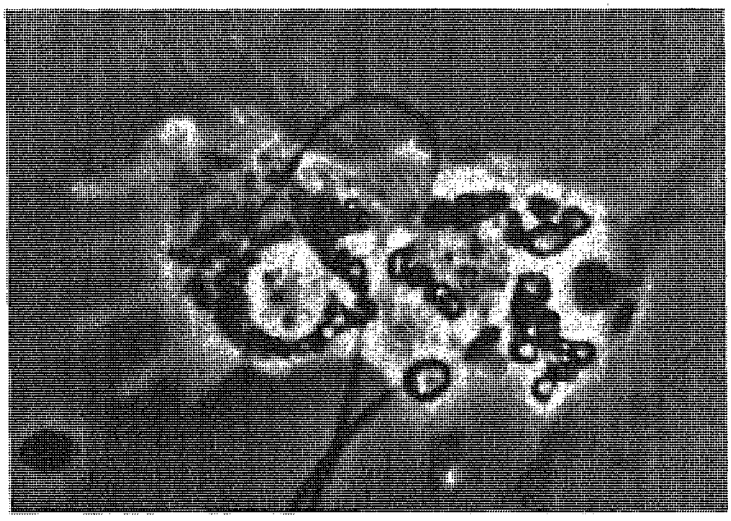

Figure 6. Living wandering cell from the blood of an earthworm immersed in a 16 per cent protein medium

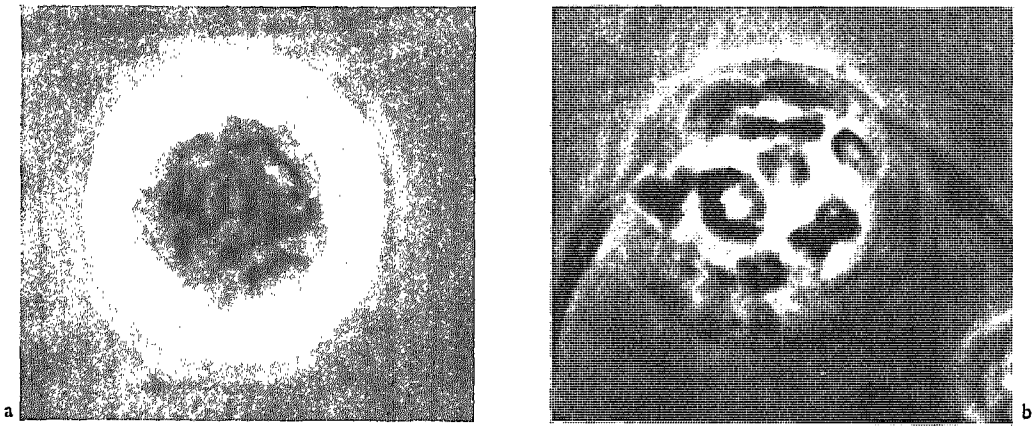

Figure 7. Locust spermatocyte: a *mounted in a salt medium;* b *immersed in a 10 per cent protein medium*

material constituting the chromosomes is capable of becoming rehydrated in an aqueous medium even after thorough drying. The concentration of the solid material is sufficiently high to form a gel whose meshes are too small to allow the protein molecules to penetrate.

The cellulose walls of plant cells have also been found to behave in a similar manner. The other parts of cells do not act like this and never become bright in protein after drying. For this reason, even though the effect depends on what is probably a non-specific physical property, it can be used very conveniently in order to demonstrate the chromosomes in non-living cell preparations. It is extremely rapid and simpler than any staining method.

Some of the points discussed here are illustrated in *Figures 6* to *10*, which were taken with a standard positive phase contrast microscope. *Figure 6* shows a living wandering cell from the blood of an earthworm. Such a cell would normally appear dark by positive phase contrast when viewed in a salt medium. Here it has been immersed in a 16 per cent protein medium and the cytoplasm appears bright. The cell was undergoing active movement.

The effect of immersion in a protein medium when viewing internal detail in living spherical cells is shown in *Figure 7*. The locust spermatocyte (*Figure 7*a) mounted in a salt medium is obscured by a halo due to the lens-like action of the cell. By immersion in a 10 per cent protein medium (*Figure 7*b) the refractive index difference between the cell and medium is greatly reduced and the lens-like action eliminated, so that the nucleus and chromosomes are revealed. Apart from the chromosomes the nucleus has a lower refractive index than the cytoplasm and appears paler.

Figure 8 shows a living insect spermatocyte in the final stages of division. The cytoplasm has been made virtually invisible by immersion in protein,

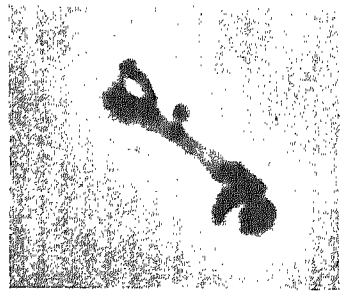

Figure 8. Living insect spermatocyte in final stages of division

and only the dark chromosomes and a bridge of mitochondria can be seen.

In *Figure 9* a dried and fixed pollen mother cell of *Tradescantia* is immersed in a concentrated protein solution. The chromosomes and the cellulose

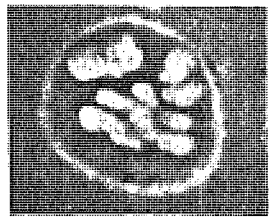

Figure 9. Dried and fixed pollen mother cell of Tradescantia *immersed in concentrated protein solution*

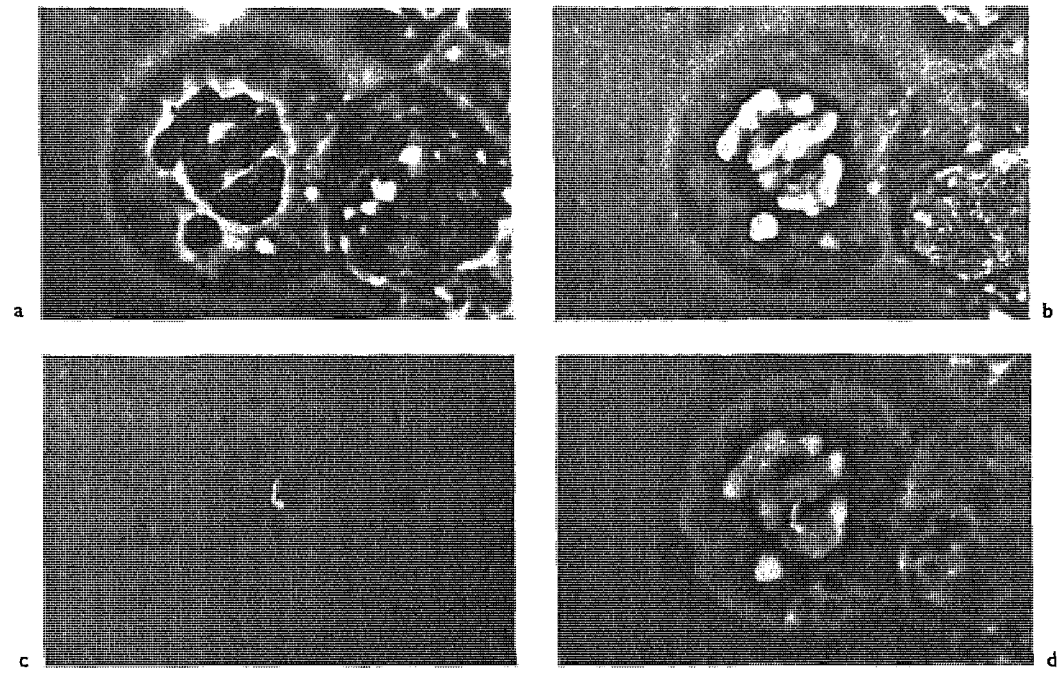

Figure 10. Salamander spermatocytes after fixation and drying, mounted in: a *water, n=1·33;* b *protein, n=1·40;* c *clove oil, n=1·53;* d *carbon bisulphide, n=1·63*

wall appear bright. *Figure 10* illustrates salamander spermatocytes after fixation and drying. In clove oil the entire cell becomes practically invisible (*Figure 10*c), but the chromosomes appear strongly reversed in contrast in a protein solution of very much lower refractive index (*Figure 10*b). The chromosomes become reversed again in carbon bisulphide (*Figure 10*d), but so does the cytoplasm, whereas in a protein medium the cytoplasm remains dark. The contrast of the chromosomes is thus greatly heightened in protein. The small bright comma-like patch in *Figures 10*c and d is a particle of extraneous debris.

CONCLUSION

In less than twenty years phase contrast microscopy has come to occupy an important place among the methods available to the biologist. Not only is it the method of choice for the routine examination of living cells, but it offers interesting possibilities as a quantitative method for measuring solid and water content of living cells. Despite the development of more perfect interference microscopes it seems unlikely that phase contrast will ever be displaced. Its simplicity and ease of operation are important advantages which make it particularly attractive for routine work.

Thanks are due to the author's colleagues, Mr S. Joseph and Miss F. M. Gaffney, who have carried out measurements and provided photographs.

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MICROSCOPIC INTERFEROMETRY

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C. Baker Ltd, London

With interference microscopes coarser features of the object can be examined than is possible with phase contrast microscopes. They avoid close restriction of the illuminated portion of the image-forming aperture and enable changes in optical path length introduced by the object to be measured.

THE broadly conceived proposal to exploit the interference phenomena which accompany the combination of two or more beams of coherent light to enhance the visibility of insufficiently absorption differentiated microscopic structures is by no means new, and probably dates back further than is often realized. Interferometers have long been in general use for the inspection, control and measurement of the surfaces of optical components, and such instruments would probably have been modified for the study of microscopic objects at an earlier date had biologists and other microscopists felt the need for such a method of investigation. But the early development of staining techniques did not encourage a search for more elaborate methods, a fact which partly explains the rather tardy acceptance of the closely allied phase contrast technique of F. ZERNIKE.

It might have been expected that the more physical applications of microscopy and, in particular, petrology would have prompted the use of interferometry, since petrologists are already accustomed to employing the interference phenomena associated with polarized light for the measurement and identification of minerals under the microscope. But the polarizing microscope, combined with immersion techniques for refractometry, has proved adequate.

The notable, widening interest in the potentialities of microscopic interferometry which now exists is partly due to a desire to overcome certain limitations which are more or less inherent in phase contrast microscopy. As is well known, the latter is a very elegant method which enhances the visibility of insufficiently absorption differentiated objects by inducing the light which they diffract to interfere optically with the light that is either directly transmitted through them or directly reflected from them. Since this is achieved by appropriately modifying the phase relationship between the diffracted and direct light components, these require to be spatially aggregated and this has proved practicable only for the relatively large amounts of diffraction produced by correspondingly fine structures and also by edges. As a result, the required selective phase modifying action of the phase contrast optical system is more complete for fine structures and edges than for coarser and more gradual ones, so that the former are misleadingly emphasized at the expense of the latter. The same selective action of the phase contrast system calls for a severe restriction in the directly illuminated portion of the aperture of the objective lens, a condition which tends to produce confusing artefacts in the image of a specimen. For many purposes these limitations are not sufficiently detrimental to warrant recourse to a more elaborate method, and the years immediately following the 1939–45 war saw phase contrast microscopy justifiably established—not only for specialized research but, subsequently, for routine investigation.

It remains to consider why those who wished to avoid the limitations of phase contrast microscopy turned their attention to microscopic interferometry. The most important reason is that, unlike phase contrast systems, the large majority of interferometers employ beam splitting devices for producing two or more beams of coherent light independently of diffraction by the object under examination. Consequently, the interference effects which result from the combination of the beams on the observer's side of the object are not restricted to the finer, highly diffracting features, but reveal the coarser and more gradual features also. Again, because there is no longer any necessity to separate the direct from the diffracted light components, many interferometers avoid close restriction of the illuminated portion of the image forming aperture. A further advantage that has been increasingly appreciated of recent years is the facility which interferometers afford for measuring changes in optical path length introduced by the object under examination, a parameter which is often usefully related to some important characteristic under investigation as, for example, the topography of reflecting surfaces or the amount of solid material in living cells.

OPTICAL SYSTEMS

The initial problem before the optical designer is to select from the considerable variety of well known macroscopic interferometers the one most suitable for inclusion in a compound microscope. Usually the best solution largely depends upon whether the microscope is intended for transmitted or incident light work.

THE ETALON AND MULTI-BEAM SYSTEMS

Probably the simplest form of interferometer for this purpose is the etalon, a well known optical device derived from the Fabry-Perot interferometer (*Figure 1*). It consists essentially of two highly reflecting yet slightly transparent, mutually parallel, flat surfaces which are spaced apart and viewed in transmitted or sometimes by reflected light. A single transparent surface which reflects light thereby functions as a beam divider, the initial incident beam being divided into a pair of beams, one transmitted and the other reflected.

In the case of the etalon the light reflected back to the light source by the first surface plays no functional part in the interferometer system, but the beam which is reflected by initial incidence at the second surface is reflected back and forth between the two surfaces, each successive internal reflection at the first surface generating a further beam which is partly transmitted through the second surface to the

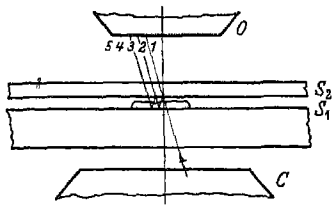


Figure 1. Etalon interferometer: C microscope condenser; S_1 and S_2 etalon surfaces; O objective

observer. Under the theoretically ideal conditions in which the surfaces would function without any light absorption and have perfect flatness, an infinite series of beams would be generated by successive internal reflection and escape to the observer through the second surface. In practice, however, there is always sufficient absorption to terminate the series and make the effective number of beams finite. This number can, however, be as large as 80 for silvered surfaces of the highest quality. Because all the beams are derived from a single original beam, their associated electromagnetic fluctuations are sequentially similar and therefore have the coherence required for the light intensity which results from their combined effect to depend upon the difference in optical path distance between successive adjacent beams. The resultant intensity for the observer is therefore a function of the separation between the two surfaces and of the optical refractile properties of the intervening medium. For example, a refractively differentiated transparent object placed between the surfaces locally modifies this effective optical separation and also the resultant intensity presented to the observer. Because the higher order, multi-reflected beams pass through the object many times, the sensitivity can be very much higher than is usual with interference systems in which there are only two beams.

Employed in the above manner, the etalon interferometer is very conveniently applicable to an existing microscope because the two surfaces can be supplied merely by depositing highly reflecting transparent films on the upper surface of the specimen slide and on the lower surface of the cover slip respectively. A practical difficulty is that the surfaces must be sufficiently durable to stand up to the usual mounting hazards and, in the case of vital cytology, must be non-toxic. These considerations tend to rule out the most optically desirable film materials such as silver and aluminium, rhodium being often preferred. Unfortunately the rather high absorption of rhodium not only reduces the available light intensity but brings down the sensitivity far below the level that would otherwise be possible, and it is doubtful whether this often usefully exceeds what can be realized with double beam systems.

Most microscopic preparations enforce a sufficient physical separation between the two surfaces to result in optical path differences of several wavelengths and it is therefore generally necessary to use a monochromatic source such as a mercury vapour lamp, usually with suitable filters to isolate the desired line.

An undesirable characteristic of the etalon is that it should be illuminated with a beam of light which is parallel, otherwise the varying angles of incidence of different rays introduce corresponding variations in optical path difference. This lack of path length uniformity corresponds to a degree of contrast which may fall to zero. For microscopic purposes a strictly parallel illuminating beam would correspond to a numerical aperture of virtually zero, and this is clearly an impossible condition which must be relaxed. The most common solution is to keep the necessary separation between the two surfaces so small that a finite illuminating aperture can be used without introducing excessively large optical path discrepancies. Even so, this aperture is usually undesirably small and, in 1947, Sir THOMAS MERTON[1] patented an ingenious system enabling full apertures to be used (*Figure 2*). In his system, the rays whose inclinations would introduce discrepant optical path differences are masked by means of concentric circular absorbing rings whose radii are proportional to the square roots of the series of whole numbers. The masking rings are in the form of a plate (usually

called a zone plate) which is fitted at the first focal surface of the condenser. A series of such plates, to different scales, is supplied to enable the one appropriate to any particular separation to be selected.

A more formidable limitation of the etalon, however, is the failure of oblique rays, leaving an object feature, to return through the same point of the preparation during the process of internal reflection.

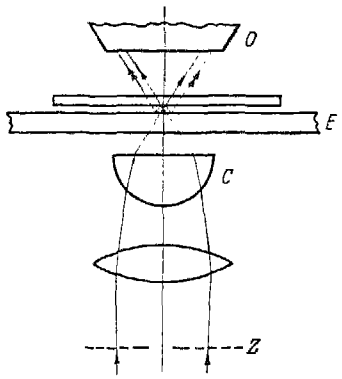


Figure 2. Merton system: E specimen-containing etalon, Z absorption-type zone plate, C condenser

This shortcoming corresponds to a limitation of structural resolution which is a positive function of the separation between the surfaces and of the obliquity (or equivalent numerical aperture). This means that inconveniently small separations must be used if full resolution is to be obtained with the highest powers.

The absence of a convenient method for measuring changes of optical path length by controlled adjustment of this parameter is a drawback which, however, can sometimes be overcome by methods such as that of R. C. FAUST[2] who has obtained valuable results with the fringes produced in a spectroscope directed towards an etalon illuminated with white light.

A particularly valuable application of the etalon to microscopic interferometry has been developed to extreme limits by S. TOLANSKY[3] for the study of surface topography. The sensitivity and precision of his method are high enough to be of the same order as a monomolecular layer on a crystal surface and it is therefore eminently suitable for the study of crystal growth. The surface to be examined is first rendered highly reflecting by evaporating a silver film upon it. One side of an optically excellent glass plate, which has been rendered highly reflective but slightly transparent, is then brought almost into contact with the specimen's surface, the plate being very slightly tilted relative to the specimen.

A microscope equipped with a visual incident light illuminator is now used to view the surface of the specimen. For reasons already given the illuminating beam must be kept reasonably parallel. In monochromatic light a series of fringes will be seen, having the appearance of sharply defined lines instead of the rather broad bands associated with normal double-beam interference. This sharply defined appearance is due to the combined effect of the large number of mutually interfering beams generated by the multiple internal reflections between the surface of the cover plate and that of the specimen, and is the condition which enables the position of the fringes to be defined with such remarkable precision. Topographical features of the specimen surface can therefore be very precisely determined by measuring the fringe deformations which they produce. Although there are the same limitations on structural resolution, common to all etalon methods, these are greatly outweighed by the immense gain in vertical resolution. However, there are more generalized applications of microscopic interferometry for which the limits of structural resolution attainable with normal microscopy must not be sacrificed and it is then usual to resort to double-beam interferometry.

DOUBLE-BEAM SYSTEMS FOR REFLECTING SURFACES WITH INCIDENT ILLUMINATION

Perhaps the earliest double-beam interference microscope for studying reflecting surfaces was that designed by W. LINNICK[4] in 1933 (*Figure 3*). In his

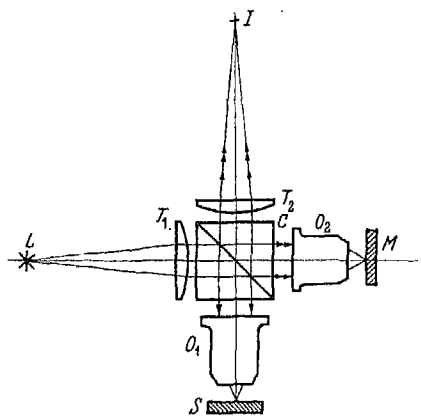


Figure 3. Basic Linnick system: S specimen, O_1 and O_2 matched objectives, M reference mirror, L lamp, T_1 and T_2 collimators, I location of real image of S and M superimposed, C beam dividing surface

instrument the specimen surface is viewed through a normal microscope objective with incident illumination provided by a semi-reflecting beam dividing surface. The beam which passes through this surface travels to a second matched microscope objective which is focused on a perfect, flat mirror. The two beams, associated with the two identical objectives, are reflected back to the beam dividing surface by the surfaces upon which they are respectively focused. The portion of the beam from the mirror which is reflected by the beam dividing surface combines with the directly transmitted portion of the beam returned from the specimen surface. The compound image formed in the eyepiece by these two combined beams therefore consists of a normal image of the specimen surface superimposed on an image of the mirror. These two surfaces are seen in interferometric comparison conditions and double-beam fringes are formed which allow the specimen's surface topography to be directly determined in terms of the effective illuminating wavelength. As no internal reflections are involved, the normal conditions of structural resolution apply. Instruments employing the same basic principle are now being increasingly used in the engineering industry for the study of finely finished metallic surfaces[5]. They tend to be costly because of the difficulties connected with maintaining sufficient stability of relationship between the two beams.

DOUBLE-BEAM SYSTEMS FOR REFLECTING SURFACES REQUIRING ONLY ONE OBJECTIVE LENS

One of the simplest systems for double-beam microscopic interferometry of reflecting surfaces is that devised by A. MIRAU which obviates the need for a second objective by including a flat semi-reflecting beam dividing surface midway between the front of the objective and the specimen surface[6] (*Figure 4*). A small central area of the front surface of the objective is locally metallized to form a miniature mirror, a reflected image of which becomes superimposed on the normal image of the specimen surface by virtue of the intervening semi-reflecting beam divider. To maintain the required degree of optical path similarity the beam dividing surface is formed on the internal side of one of a pair of identical plates which are cemented together.

Earlier, in 1951, J. DYSON[7] described a basically similar system, instrumentally elaborated to include his long working distance device. The latter enables any objective to be used, irrespective of working distance, whereas the simpler Mirau system is confined to objectives having adequate working distances. In Dyson's system the semi-reflecting surface and the small metallized area are formed on

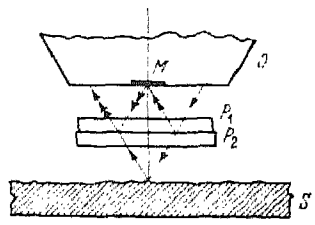


Figure 4. Mirau system: O objective, M mirror, P_1 and P_2 plates, S specimen

glass blocks. The whole system is brought into oil immersion contact with the specimen surface to maintain sufficient equality of optical path between the two beams. This means that the surfaces of dielectric specimens must first be metallized, a disadvantage which does not apply to the Mirau system.

DOUBLE-BEAM SYSTEMS FOR TRANSPARENT SPECIMENS IN TRANSMITTED LIGHT

It might appear that any of the incident light systems mentioned above could equally well be used for studying transparent objects by replacing the normal reflecting specimen with a good surface mirror on which the transparent object is mounted under a cover slip. For the lowest powers this is probably the case but even moderate powers raise difficulties. The cover slip introduces spherical aberration with a dry objective, and disturbs the required similarity of optical path between the two beams. With the more elaborate double-objective type of system this might be overcome by replacing both objectives with another matched pair corrected for cover glass and placing identical cover glasses in front of both. A similar result for a Mirau system might be achieved by providing another beam dividing pair of plates with a small piece of cover slip cemented to the upper surface. The difficulty does not apply to the Dyson system because of the immersion condition. A more fundamental obstacle arises from the finite separation between the surface of the supporting mirror and an object feature which may not be in contact with it. This results in a second return image from the mirror which cannot be focused coincidentally with the primary image and therefore leads to loss of resolution except for features which are virtually in contact with the mirror. For these and other reasons which there is not space to enumerate here, transparent objects have called for specially designed systems.

TRANSMITTED LIGHT SYSTEMS

After the example of the Linnick type of system, an obvious device would seem to consist of a corresponding transmitted light version, having a pair

of matched condensers to illuminate the two objectives so that the specimen slide could be supported in one beam without disturbing the second beam. The two condensers would be illuminated by two beams from an initial beam divider to be recombined at a second beam dividing surface after leaving the objectives. Unfortunately, such a system must be considered too cumbersome and inherently unstable to be a practicable scientific instrument.

A more practicable system can be devised if both beams are permitted to pass through the specimen area. This is permissible for true interferometric purposes, provided that the separation in the specimen area between corresponding portions of the two beams exceeds the distance between a particular object feature and an unoccupied portion of field serving as reference area. This was appreciated as early as 1930 by A. A. LEBEDEFF[8] who constructed a double-refracting plate form of instrument designed for low power work. In 1947 the author[9] independently stumbled on the same basic principle and designed a series of different systems exploiting it in different ways: two of these will be described later.

SYSTEMS IN WHICH BOTH BEAMS ARE DIRECTED THROUGH THE SPECIMEN AREA WITH THE AID OF SEMI-REFLECTING BEAM SPLITTERS

In 1948, J. ST. L. PHILPOT[10] disclosed a number of systems having semi-reflecting beam dividers and combiners. In one of these—the cyclic system (*Figure 5*)—the two initially divided beams are conducted respectively in opposite directions through an identical pair of objectives on a common axis, one of which functions as a normal substage condenser. The required beam separation in the object space between the two objectives is achieved by slightly defocusing one of the objectives. After passing through the two objectives successively, the two emerging beams return on the same closed path to the beam divider where they are combined by the usual process of partial reflection and transmission. The beam which leaves the microscope system through the focused objective provides a correctly focused image of the object, but the other beam leaving through the defocused objective provides a superposed out-of-focus image of the object. Consequently, the final image presents an interferometric comparison between a true image of the object and a smoothed defocused image of it. All systems exploiting this basic principle of focal difference are therefore known as double-focus ones.

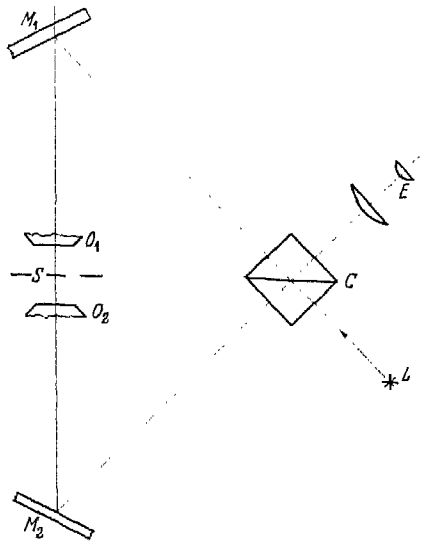


Figure 5. Basic cyclic system by Philpot: L lamp, C Swann cube, M_1 and M_2 mirrors, O_1 and O_2 objectives, S specimen, E eyepiece

The large amount of beam separation required to direct the two beams through a microscope system from opposite ends calls for a specially designed stand having a degree of stability not easy to realize in practice, and this tends to offset the advantage gained by permitting both beams to pass through the specimen area.

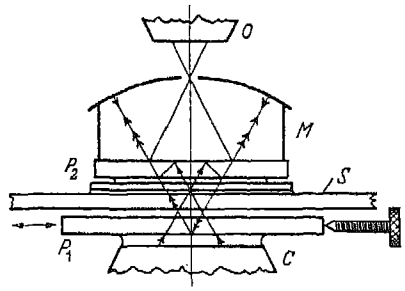


Figure 6. Dyson system: C condenser, P_1 and P_2 wedge-shaped plates, S specimen, M spherical mirror, O objective

This difficulty was avoided in a double-focus system of a less elaborate kind described by J. DYSON[11] in 1949 (*Figure 6*). In this system, the initial transparent beam dividing surface is formed on the upper side of a glass plate mounted normally to the optical axis of the microscope immediately beneath the specimen slide. The illuminating beam, directed convergently through this upper surface by the substage condenser, is then partially reflected back to the lower surface of the plate which is partly metallized. The lower surface of the plate

therefore reflects this second beam back through the upper surface of the plate. As a result, the specimen area is illuminated by two beams, one of which is focused on the specimen after direct transmission through the plate while the other reaches the specimen in a defocused condition due to internal reflection within the plate. A similar plate between the specimen and the objective lens functions in much the same way, so that the portion of the second defocused beam which passes directly through it becomes combined with a portion of the first focused beam internally reflected within it.

The image formed by the microscope objective consequently consists of a correctly focused image of the specimen area seen in interferometric comparison with a strongly defocused image of it. Dyson's long working distance reflecting device is included between the second plate and the objective to allow medium and high powers to focus through to the specimen. The two plates are made slightly wedge-shaped so that the optical path difference can be manually adjusted by traversing the condenser plate (with reduction lever and micrometer screw) in a direction parallel to the principal section of the wedge across the optical axis of the microscope. This operation varies the effective thickness difference between the two plates and thereby controls the optical path difference. Theoretically, the objective plate can combine perfectly only when the plates have identical thicknesses, but in practice there is sufficient latitude for adequate range of variation.

The two superimposed fields can be slightly tilted to produce a system of straight interference fringes in the field by rotating the objective plate about the optical axis so that the two wedges no longer compensate each other, the number of fringes being limited by the wedge angle. Since recombination occurs only when the plates are optically parallel, the substage plate is provided with a tilting adjustment. The relatively large amount of beam separation in the specimen area calls for a high degree of optical homogeneity which is achieved by bringing both the condenser and objective plates into oil immersion contact with the slide and cover glass of the preparation. Non-functional reflections from the semi-reflecting surfaces produce ghost images which appear to rule out the use of low powered objectives, a limitation which, however, is often unimportant. For medium and high powers these reflections are lost and their only effect is severely to reduce the effective light transmission. In spite of such minor disadvantages the Dyson system is the most successful of known semi-reflecting systems.

DIFFRACTING SYSTEMS

Another method of beam division and combination is provided by the familiar phenomenon of optical diffraction (*Figure 7*). A small structural element, illuminated by a light wave, generates a secondary wave, so that a regular periodic arrangement of similar elements generates a regular series of secondary waves which coalesce to form diffracted light beams which diverge from the original illuminating beam. The finer the diffracting structure relative to the wavelengths of light, the more the diffracted beams diverge from the original illuminating beam which usually passes straight through the structure and is called the zero order. Such a beam dividing structure may be referred to as a diffraction plate. In its theoreticallys implest form it may consist of a regularly spaced system of similar apertures in an opaque screen (amplitude type) or of grooves on the surface of a transparent plate (phase type).

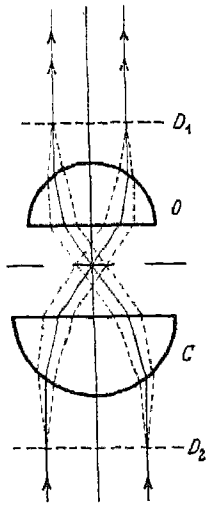


Figure 7. Basic diffracting system: C condenser, D_1 and D_2 diffraction plates, O objective

In 1948 the author[9] devised some systems employing diffracting beam dividers and combiners in which the diffraction plates had one of the following forms: normal diffraction grating consisting of equi-spaced straight lines, zone plate consisting of concentric circles whose radii are proportional to the square roots of the series of whole numbers, uniformly spaced concentric circles and, finally, a spiral. The beam dividing diffraction plate is located at the first focal surface (near the normal iris diaphragm) of the substage condenser of the microscope and the second optically identical plate at the back focal surface of the objective lens. The two diffraction plates are then conjugate and the second will bring

about recombination, provided that it is in optical register with the real image of the corresponding plate in the substage. Under these conditions the direct zero order beam passes straight through the object and the second diffraction plate, and is therefore focused to a true image by the objective lens. The light which is deviated by diffraction at the substage plate, however, is thereby deflected away from the object, but again meets the direct beam at the back focal plane of the objective where the second plate is located. Each element of the second plate is therefore illuminated coherently by both beams, so that the resulting secondary waves coalesce to effect a combination of the direct and diffracted beams. A disadvantage is that contrast is reduced by non-functional diffracted beams generated by the diffraction plate of the objective unless the illuminated area of the specimen is small compared with the size of field. Again, it is difficult to provide adjustment of optical path difference.

A theoretically possible solution to the latter problem depends upon the use of a special form of diffraction plate consisting of grooves formed in the surface of a plate made from double-refracting material. Being birefringent, the plate has two refractive indices so that, when its grooved surface is joined to a glass cover plate with a cement whose refractive index matches one of its own indices, it will be equivalent to a uniform glass plate for the corresponding refractive index but retain its diffracting properties for the second refractive index.

Since the two refractive indices of a birefringent substance are usually associated with polarization in corresponding perpendicular planes, it becomes possible to adjust the optical path difference by using a variable birefringent compensator, such as is often employed with polarizing microscopes. It is still necessary, however, to suppress the zero order beam which is normally generated by the diffraction process. In principle this can be done in one of two ways, namely by giving the grooves a suitable prismatic form (blazed) or by giving them a depth such that the optical path difference between surface and groove is one or more odd integral numbers of half-wavelengths. In the latter case the secondary waves destructively interfere in the zero order direction so that only the higher order beams appear. It then becomes possible to use the birefringent compensator to adjust the optical path difference between the direct light for which the special plate functions as a piece of plane glass and the diffracted light which is devoid of the zero order. Only one of the two plates need have this special form and it is therefore usually preferable to have the special one in the substage, so that the objective plate (which may have to be on a lens surface inside the objective system) may be a photographically formed absorption type. At present, however, it seems unlikely that the special form of plate could be made, at any rate under commercial conditions.

An interesting development of diffracting systems is due to J. M. BURCH[12] who has pointed out that it is unnecessary for the two diffracting structures to be regular, provided that they are identical and accurately registered relative to each other. The only essential difference is that a random structure results in a correspondingly random distribution of the diffracted light. In practice the second plate is a strip replica of the first. Since random structures (formed, for example, by turning or grinding) are likely to contain at least a proportion of very fine elements, it may be found difficult with the higher powers to image one structure upon the other with sufficient perfection for good contrast.

DOUBLE-REFRACTING SYSTEMS

A method of beam division and combination which is particularly applicable to interference microscope systems depends upon the interesting phenomenon of double refraction. Dielectric crystals, other than those belonging to the cubic system, have a directional form of lattice structure which imparts refractive properties that are also directional. For instance, in an important group of crystals of which quartz and calcite are familiar examples, a light wave entering the crystal is divided into two waves, one spherical and the other ellipsoidal. These two waves have a common axis of rotation at which they usually touch, known as the optical axis of the crystal. In all directions other than that of the optical axis the separation between the two waves resulting from the ellipsoidal form of the second extraordinary wave corresponds to a difference in velocity (and so of refractive index) which reaches a maximum for any direction perpendicular to the optical axis. In general, then, there are two refractive indices, one for the spherical ordinary wave which is independent of direction and the other for the extraordinary wave which varies with direction.

Because of this property, an optical component made from a birefringent crystal refracts an incident beam along two different paths, thus dividing it into two beams, provided that the direction of entry is not along the optical axis of the crystal. Generally the two beams are polarized in perpendicular planes. It is also possible to recombine two such beams by a suitably shaped optical component made from a birefringent crystal whose refractive indices cause the beams to be differently refracted into coincidence. Provided that the polarization

direction of the second, combining component corresponds with those of the two beams, there are no parasitic beams to mar the contrast or to reduce the light intensity, such as tends to be the case with combiners of the semi-reflecting type.

For the two beams to interfere, however, it is essential to place the two double-refracting components between a polarizer and an analyser so that the effective transmission rarely exceeds 30 per cent.

Macroscopic interferometers exploiting double refraction have long been known[13] but very little used because of the difficulty of obtaining adequate beam separation. For microscopic interferometry, however, this is not a serious drawback because the small dimensions of most microscopic objects do not require large amounts of separation.

These considerations led the author[9] to devise a series of double-refracting interference microscope systems, two of which are direct transmission types for transparent specimens. One of these (*Figure 8*) employs lenses made from birefringent crystals associated with the condenser and objective systems of the microscope, to impart a double-focus effect on the two beams generated by the birefringence. The two lenses must be in conjugate positions, preferably at the front focal surface of the condenser and the rear focal surface of the objective respectively. For the higher objective powers these surfaces are within the lens train of the objective and it becomes necessary to compute a special objective having a birefringent lens component at the appropriate position within the train.

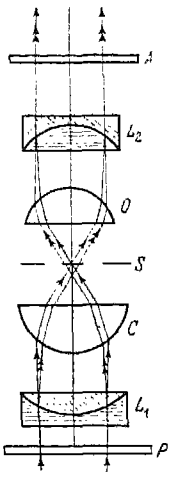


Figure 8. Smith double-focus system: P plate, L_1 and L_2 birefringent crystalline lenses, C condenser, O objective, A analyser plate (optical path length adjustment can be effected by a variable birefringence compensator anywhere between P and A)

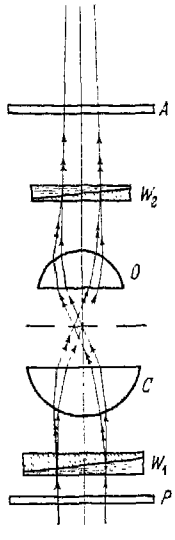


Figure 9. Smith shearing system: P polarizer, W_1 and W_2 birefringent crystalline prisms, C condenser, A analyser

In the alternative system (*Figure 9*) the two birefringent members have a prismatic form, so that the double-refracted pair of beams from the substage member mutually diverge to be brought to foci which, instead of being concentric and axially separated, are co-planar and separated laterally. Systems in which the beams are separated in this lateral sense are known as shearing systems to distinguish them from the double-focus types.

With prismatic systems it is not essential for the birefringent members to be conjugate because the amount of double refraction is constant over the entire aperture of the prism. On the other hand, the divided pairs of rays generated by the double refraction must intersect in a surface which is concentric with the front and rear focal surfaces of the condenser and objective respectively. For low powers these surfaces are sufficiently flat to obviate any difficulties due to this requirement, but with the higher powers it must be met either by suitably curving the two prisms or by introducing the required curvature by steeply curved intervening lenses.

Although it is not essential for the prisms to be conjugate, departure from this condition results in a field which is crossed by straight interference fringes whose spacing is virtually inversely proportional to the departure from conjugate condition. These can, however, be broadened and even entirely 'fluffed out' by, for example, introducing a birefringent wedge of appropriate angle in the plane of the visual field.

There is an alternative and instrumentally simpler class of double-refracting systems in which the birefringent beam dividers and combiners are plates that are made to exert either the double-focus or sheared form of separation by appropriately selecting the direction of the optical axis of the crystal in relation to the surfaces of the plate. The two divided component rays in each pair produced by a birefringent plate emerge mutually parallel, so that a second plate can recombine them irrespective of its distance from the first plate. In other words the plates need not be conjugate. This makes it possible to include the plates between the condenser and the objective with the specimen between the plates.

In the case of the double-focus type of beam separation the two plates are cut with their surfaces

parallel to the optical axis of the crystal because for this direction the two wave fronts proceed in the same direction but have different curvatures. To obtain sheared separation, however, the plates are cut obliquely to the optical axis because the two wave directions are then mutually divergent.

Whatever the selected axis direction may be, fairly rigorous conditions must apply for the objective plate to recombine the two separated beams from the plate of the condenser. The directions of their optical axes must be identical, the plates of the objective and the condenser must be of equal thickness, and instrumental means must be provided to maintain sufficient optical parallelism between both plates. Further, some means must be provided to ensure that the beam whose wave fronts are spherical (ordinary) in the plate of the condenser are ellipsoidal (extraordinary) in the plate of the objective and *vice versa*, otherwise the resulting wave fronts will lack the identical shape essential for combination.

The last condition can be attained in one of two ways. In the first, both plates are made from one single plate and are therefore identical in thickness and in optical axis orientation. The required exchange between the ordinary and extraordinary beams is then brought about by introducing between the plates a birefringent half-wave retardation plate. The effect of the half-wave plate is to rotate the plane of polarization of an incident beam and, for the present application, it is made to produce the required 90° rotation by orientating it with its polarization directions in a diagonal position.

In the second method the two plates are made from different types of crystal whose birefringences have an opposed sense. Under these conditions the wave which is extraordinary in the first plate is also extraordinary in the second, but in an opposite sense, so that the two opposed shapes tend to counterbalance and give a resulting form which is nearly spherical. Since, however, most available crystals, such as quartz and calcite, which have opposite signs of birefringence usually differ also in their refractive properties precise compensation is not achieved. In practice this can be overcome by obtaining the small amount of remaining compensation from a birefringent lens located at the first focal surface of the condenser. Both these methods have specific advantages and are employed in the double-refracting instrument made by C. Baker Ltd (*Figure 10*).

In this instrument the substage polarizer is provided with a 45° rotation, one extreme position extinguishing the second, interfering beam to give normal transmitted light illumination, the other giving both beams with equal intensity. The latter condition provides the best contrast when there is negligible absorption, but if appreciable absorption is present its attenuating effect on the object beam can be met by rotating the polarizer a suitable amount towards the single beam position. The polarizer mount can also be swung clear of the

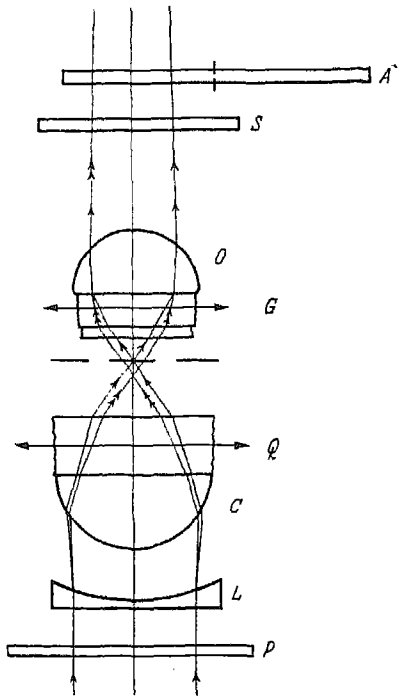


Figure 10. Baker double-focus immersion system: P polarizer, L birefringent lens, Q quartz plate, G glass protected calcite plate, S birefringent quarter-wave slide, A rotatable analyser

optical axis when it is desired to use normal objectives without polarized light.

Various birefringent compensators may be used for the measurement of optical path difference, but a simple Senermont system, consisting of a birefringent quarter-wave compensator plate followed by a rotatable analyser, is built into the body of the instrument. Both the quarter-wave plate and the analyser have an ' out ' position to permit alternative methods to be employed. For example, fringe field conditions can be obtained by replacing the normal ocular tube with a simple form of slotted eyepiece provided with a quartz wedge. An alternative half-shadow principle eyepiece[14] is also available for increasing the accuracy with which visual measurements can be made.

SYSTEMS EMPLOYING ONLY ONE BIREFRINGENT MEMBER

It is possible to simplify birefringent systems by replacing one of the two birefringent members with a diffraction plate designed optically to match the other number. Since, however, the effect of a diffraction plate is a direct function of wavelength such systems have the disadvantage of requiring monochromatic illumination. This can be avoided by using a system described by M. FRANÇON[15,16] in which the diffraction plate is merely an appropriately shaped narrow aperture in a substage diaphragm near the first focal surface of the condenser. Such an aperture does not divide the initial beam into two beams but spreads it out by simple diffraction so that the object is coherently illuminated. Although he describes a plate system to recombine the light from different elements of the specimen area, any other equivalent type of number may equally well be used. The substage aperture is a slit for shearing systems and an annulus for double-focus ones. The double-focus effect, however, is obtained by cutting the crystal plate with the optical axis parallel to that of the microscope, a condition which the author has been unable to employ because of a theoretically interesting reduction of resolution caused by the combined action of different planes of polarization which this direction includes.

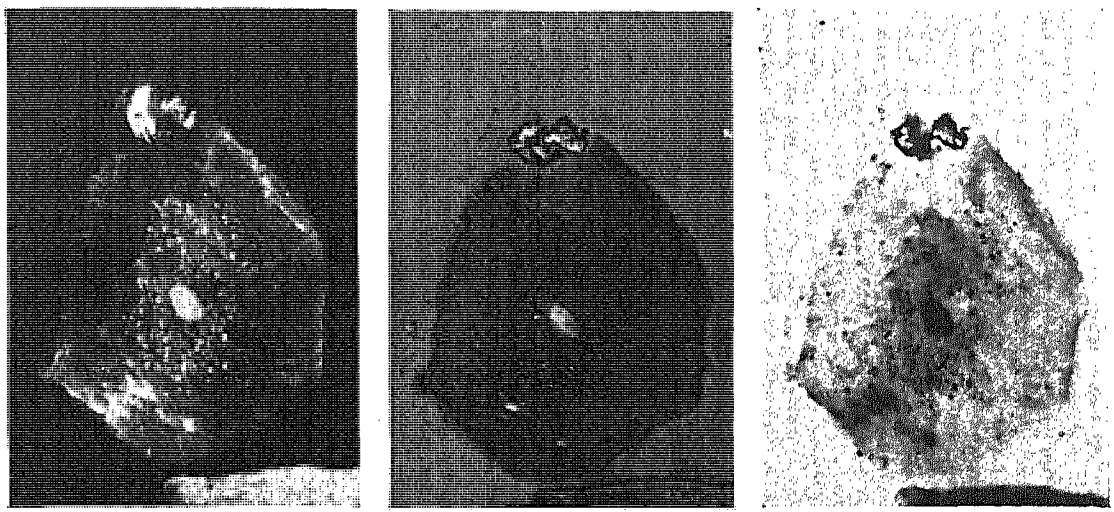
Systems of this diaphragm type suffer from the fact that the maximum allowable beam separation associated with the birefringent member is limited by the narrowness of substage opening which it is practicable to use. Usually this has to be too small for the larger living cells to be seen with the higher powers in interferometric comparison with a reference area outside themselves.

It can, however, be argued that, although possibly desirable, such separation is not essential because with a separation too small to be discerned by the eye the resulting interference field gives the derivative of the optical path profile. It may, unfortunately, prove difficult to perform the integration required to obtain the true optical path profile, especially with objects of somewhat irregular shape such as many types of cell. Furthermore, the differentiation applies to only one direction (shearing system assumed) and a second set of measurements would therefore have to be made with a relative rotation between the shear direction and object.

At present it would appear that systems with infinitesimal beam separations are preferred only where their simplicity is more important than the direct form of measurement possible only with systems giving substantial separation.

APPLICATIONS

Practical double-beam transmitted light instruments have only recently become commercially available and it would be rash, at this early stage, to attempt to formulate all the most likely applications. Up to the



a b c

Figure 11. Unstained cell from oral epithelium in saliva: a *with phase control adjusted to darken the surrounding medium;* b *with control re-adjusted through 96°, a setting which caused the cytoplasm to darken;* c *with control re-adjusted through 204° to darken the nuclear region (photographed by A. Barron with a* ×40 *shearing objective and reproduced by courtesy of C. Baker Ltd)*

present, one of the important applications has been to the study of living cells (*Figure 11*), partly because this method yields images which are more directly related to substantial structure than is the case for such earlier methods as dark-ground and phase contrast illumination. A more important reason is the possibility it affords for the determination of the concentrations of solid material within a cell and also of the total quantity present, both being possible while the cell is alive.

Both these important determinations[17,18] depend upon the fact that the increase in the refractive index of cytological material in solution is substantially proportional to increase in concentration. The precise rise in refractive index due to a concentration increase of 1 per cent does not differ by more than 10 per cent for any of the chemical substances contained in cytological material and is usually given a mean value of 0·0018.

This linear relationship enables the concentration of the combined solid substances to be determined from the refractive index of the living cytological material with an error not exceeding 10 per cent.

Since the optical thickness of the object (change of optical path length) is the product of refractive index and physical thickness, the refractive index of the object is readily calculated if its physical thickness and the refractive index of the surrounding medium are known. The latter may be measured with any convenient form of refractometer or with a specially designed interferometric cell mounted on the stage of the interference microscope. The thickness of the object is usually more difficult to determine because of the inherent irregularity of cytological specimens. One solution is to compress them with a descending cover slip by a known amount. This may be ascertained from interferometric measurements on nearby air bubbles in the preparation.

In an alternative method described by R. BARER[19] it is unnecessary to know the thickness, the required refractive index being calculated from two optical path measurements made respectively in two immersion media having different, known refractive indices. Conversely, the thickness can also be calculated in a similar way. This double immersion method presents the cytologist with the problem of preparing and introducing a second medium having a usefully different refractive index which will not interfere with the complex processes of the living cell, but it appears that this can often be achieved.

Determination of the total quantity of solid material contained in solution in the cell also depends upon the same known linear relationship between concentration and refractive index. Provided that the surrounding reference medium is water, any local optical path change inside the perimeter of the cell is proportional to the equivalent thickness of solid material presented to the corresponding portion of the object beam. Integration of the various optical path lengths presented by the cell therefore permits a calculation of the mean equivalent thickness of solid material present. The product of this calculated thickness and the area of the cell then gives the volume of dry material. A particularly valuable feature of both these determinations is the information they can provide concerning the processes of cell growth.

Very beautiful and informative cinematographic films in colour, which show the movements and changes of living cells under speeded up (time lapse) conditions, have recently been made, using a standard commercial double-refracting instrument. Although most cells are virtually colourless they can be made to appear coloured under interference conditions in white light because the simultaneous presence of different wavelengths results in a corresponding mixture of phase differences. Since intensity is a function of phase difference some wavelengths are attenuated in relation to others and colour contrast is thereby obtained. The colours are related to optical thickness in a sufficiently direct way to be informative to an experienced user. It will be interesting to observe the development of new applications and techniques which the recent advent of commercial instruments may stimulate.

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FLYING SPOT MICROSCOPE

Use in Particle Analysis

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The flying spot microscope has already been applied for counts of red cells, nerve cells, and other biological and metallurgical materials. In this article, the method is outlined, and the counting of total numbers of particles of all sizes, as well as the size distribution of particles is discussed.

THE NEED for a machine that will estimate the number and sizes of particles in a microscopic field is felt in many investigations. The method that uses scanning of the microscopic field by a small spot of light produced by the combination of a standard microscope and a conventional television flying spot scanner cathode ray tube has proved to be both practicable and versatile. With suitable modifications it will probably meet many requirements. Besides its use for the study of microscopic fields of biological and metallurgical material, it can also be applied to the examination of photographic plates and film, for example electron micrographs and track photographs. The principles of the machine, and the types of information obtainable, have already been described[1,2] elsewhere.

DESCRIPTION OF COUNTING EQUIPMENT

It is convenient to use a system that allows for two speeds of scanning. A high speed provides a good visual display, but a low speed is used for counting and sizing, since it gives a better signal-noise ratio and allows for simplification of the counting equipment. *Figures 1* and *2* show the general layout of the system, including the split-beam arrangement that is used for counting and sizing.

The time base generator and scanning tube produce a scanning spot of light that passes into the eyepiece of the microscope and then through the objective, specimen, and condenser on to a photocell. Thus the specimen is scanned by a spot much smaller than the particles in the field. As the spot crosses opaque areas in the specimen, pulses are generated and passed on to an amplifier whose output controls a display tube. The latter is operated from the same time base generator as the scanning tube and thus records an image of the specimen.

For counting and measuring, the preparation is scanned not by one but by two spots, one line width apart, recording on separate photocells. The output from the amplifiers is fed to an anti-coincidence

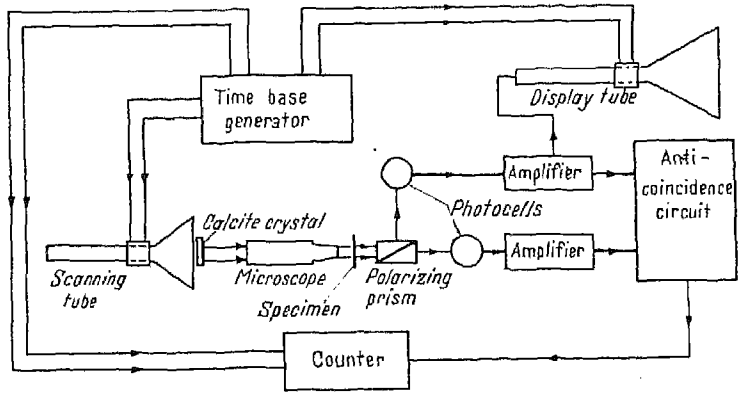

Figure 1. Schematic diagram of flying spot microscope

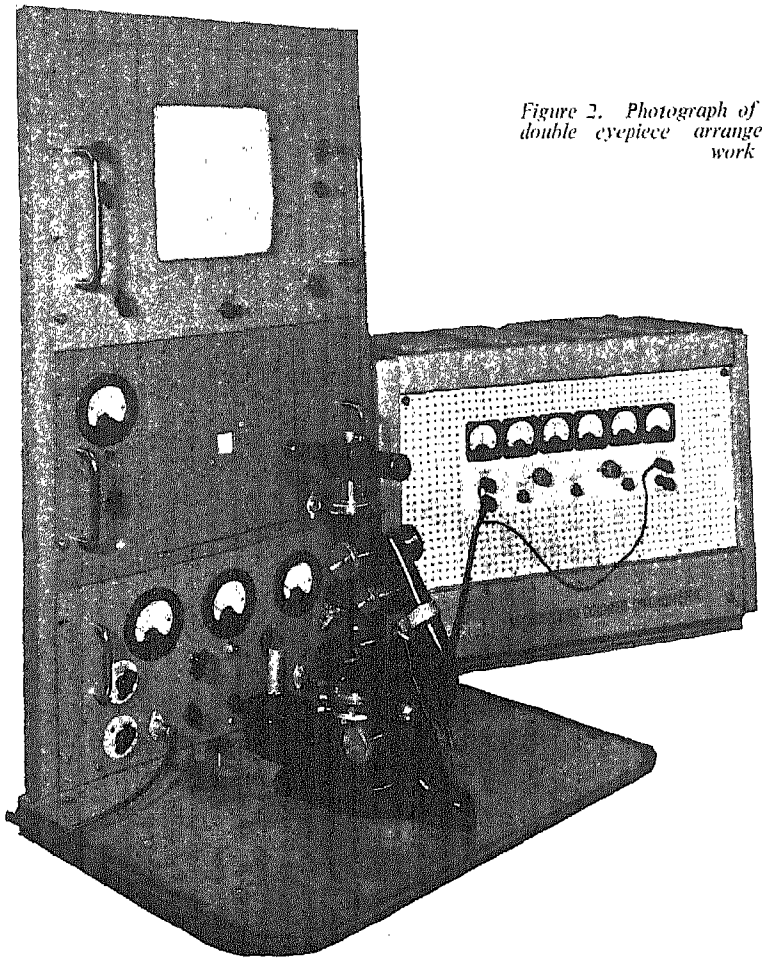

Figure 2. Photograph of machine showing double eyepiece arrangement for visual work.

circuit, which is arranged to give an output only when one of the spots is obscured but not the other. This will obviously occur twice during the scanning of a particle of spherical shape, once as the scans first reach the particle and again as they leave it (*Figure 3*). It is arranged that one of these pulses is passed to a counter. In this way the total number of particles of all sizes is recorded. Particles of certain shapes with large re-entrants may be counted twice, but in practice this situation has been seldom observed.

In order to provide two beams one line width apart, the scanning beam is passed through a birefringent crystal. Beams polarized at right angles to each other and separated by a distance proportional to the thickness of the crystal are thus provided and can be separated widely by means of a polarizing prism placed below the condenser. Each beam is thus passed to a separate photocell and the preparation is scanned by two spots.

An alternative method by which the double spot scan can be simulated is to use a one-line memory system and to arrange that a count is recorded only if the spot is not obscured at the corresponding place on the previous line. This system avoids the complications involved in balancing the output of the two photocells in the anti-coincidence circuit.

COUNTS OF BLOOD CELLS, NERVE CELLS AND DUST PARTICLES

This method has been applied to counts of red cells, nerve cells, dust particles and other materials. The results of counting a series of red cell samples are shown in *Table I*. Each sample was counted by two different human observers and then twice by the machine[3].

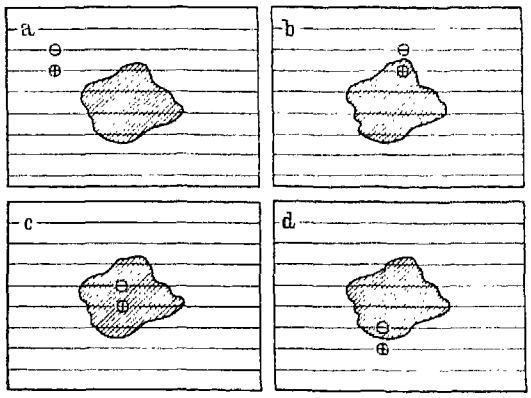

Figure 3. Counting by means of double spot technique

From these results it appears that the counts by the machine are no less consistent than those of human observers, and they may be more so; further work is required to show this. The machine counts are, of course, very much quicker than the visual ones. The time taken to prepare the specimen is the same in the two cases but the machine counts in 4 seconds as against at least 4 minutes (often much more) for the human observer.

Table I. Numbers of Red Cells (M/mm³)

Sample	Visual		Machine	
	1	2	1	2
1	3·9	3·8	3·9	3·8
2	5·1	4·4	4·5	5·3
3	4·1	4·0	3·7	4·0
4	2·5	2·4	3·0	3·0
5	1·2	1·1	1·3	1·3
6	3·2	3·1	3·0	3·3
7	3·6	3·7	3·5	3·5
8	3·5	3·8	3·5	3·6
9	3·9	3·8	3·7	3·7
10	4·5	4·5	4·8	4·6
11	4·8	4·7	4·6	4·6
12	4·7	4·4	4·4	4·4
13	4·9	4·8	4·7	4·7
14	4·6	4·7	4·7	4·7
15	3·3	3·3	3·4	3·3
16	6·7	7·0	7·1	6·2
17	4·2	4·1	4·1	4·1
18	4·5	4·3	4·2	4·2
19	4·2	4·2	4·2	4·2
20	5·2	5·2	4·9	5·0

An example of counting in sections of biological material is provided in *Table II*, where machine counts of all the particles in a stained section of rat's cerebral cortex are compared with counts of the number of nuclei as made by a human observer. Each count is for a strip of cortex 500μ wide, extending from the brain surface to the white matter. The machine counts are mostly slightly larger, because they include stained particles that are not nuclei, which are rejected by the human observer. However, the loss in accuracy is not great considering that the machine counts are made in a few minutes, whereas the human observer takes many hours.

A further example is the counting of dust particles which is particularly difficult since often many are near and below the limit of resolution. *Table III* shows the results with a series of thermal precipitation samples of steel dust. These were kindly supplied by G. M. MICHIE, of the British Steel Castings Research Association, and were counted independently by human observers in Sheffield and London (columns 1 and 2). Replicated machine counts (columns 3 and 4) agree better with each other than do the counts of the human observers, but these latter had not consulted over standards.

There is therefore good reason to think that this method of counting can be applied to various types of particles, that it gives results not greatly different from those of human observers, with a consistency which is not widely different from the human but may be considerably greater.

SIZING SYSTEM

The ideal method for obtaining the size distribution of particles would be one in which each particle is treated separately. For example, having identified the leading edge of the particle by use of the double spot system, the original scanning system could be stopped and an auxiliary scan introduced to scan over only the particle recognized. The number of times the double spots cross and the total length of intercepts could then be recorded, giving the total area of the particle.

Although this method is probably the only satisfactory one for dealing with irregularly shaped particles, the equipment necessary for consecutive particle analysis would be rather complex. A much simpler method has been described[4], in which the maximum dimension of the particle in the direction of scan is taken as the measure of the size of the particle, the error being subsequently reduced by taking successive scans with different orientations of the field. In this method a pulse *dt* of known duration is generated by the leading edge of each intercept. This pulse *dt* is then compared with the length of the intercept. Only those intercepts whose duration is greater than *dt* are effective in the counting circuits.

Table II

Total particles: machine count	*Nuclei: visual count*
1336	1253
1183	1220
1172	1303
1164	1181
1410	1424
1044	1389
1197	1170
1296	1357
1464	1503
1389	1357
1289	1242
1018	1048
1315	1258
1240	1104
1349	1087
1372	1069

For particles whose maximum intercept is less than dt no count is registered. Thus when dt is zero all particles are counted, and as dt is increased only successively larger particles are counted until dt is greater than the maximum intercept of the largest particle, when the count is zero. Successive scans are made with various durations of pulse dt and a cumulative distribution curve is obtained.

Figure 4 shows how the wave form obtained from each line is modified. For example, 5_C represents the output from the photocell on line 5, after it has passed through a clipping amplifier, that produces a rectangular wave form from the portions of the signal above a given amplitude. A second wave form 5_T goes positive at the same time as 5_C but returns to zero after a time dt, or when 5_C returns to zero, whichever is shorter. The value of dt can be varied in fixed steps. A third wave form 5_S is obtained by subtracting 5_T from 5_C and is thus the wave form that would be produced by the photocell if only the parts of the particles enclosed by the dotted lines and the right-hand edges were present. This wave form is then differentiated at 5_D. Due to the double spot system described earlier, wave forms 5_D and 6_S are produced simultaneously. The A pulses of 5_D are made to change the state of a condenser or two-state storage device, and the B pulses to restore the original state and produce an output pulse to the counter. Any portion of wave form 6_S is also able to restore the state and if it does so before B occurs there is no count. The effect of this is to count only the last intercepts of particles having a portion longer than is represented by the given time dt. Thus for lines 5 and 6 there

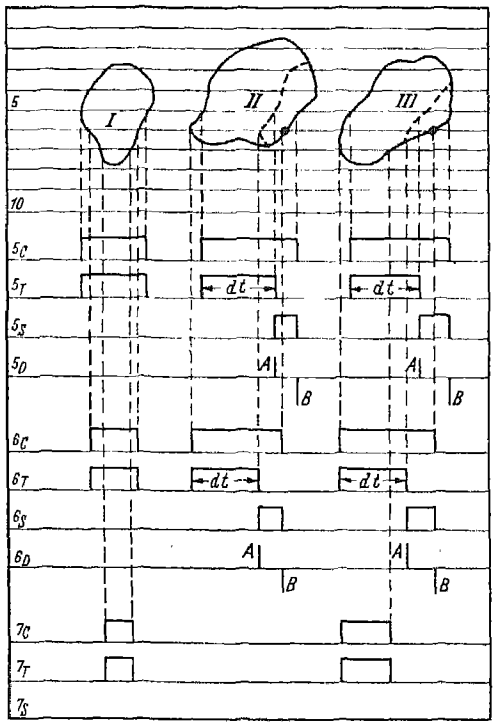

Figure 4. Wave forms for particle size distribution

Table III. Steel Dust Particles: Total Number of Particles per Strip (All Sizes) $\times 10^3$

Visual		*Machine*	
1	2	1	2
497	525	490	510
513	475	522	496
506	476	639	520
928	496	869	792
862	682	714	804
334	320	354	350
1045	869	765	810
599	550	515	487
349	385	362	405
516	473	494	484
818	784	898	860
640	675	590	620
309	315	374	348
639	475	560	496
416	386	540	492
667	435	395	463
316	343	375	393
615	555	492	569
951	510	365	400
461	420	432	392

is no count, but counts will occur for lines 6 and 7 because particles II and III produce no wave form 7_S on line 7 and the changed state of the storage device produced by pulse A, 6_D, is returned to its original state by pulse B, producing counts.

This system will operate accurately except for particles with large re-entrants, which will be counted more than once. The method, of course, gives only the dimensions of the particles in the direction of scan. If dimensions in other directions are needed the orientation of the preparation in relation to the scan can be changed.

The method has been used to measure particles of various sorts. *Table IV* gives the results with particles

Table IV

μ	*Machine*	*Human observers (mean)*
0–5	184	189
6–10	136	137
11–15	45	46
16–20	20	26
21–25	13	10
26–30	8	8
30	2	2
Total	408	418

of a paint spray counted by the machine and by three visual observers, using a photograph of the same field.

MARKING OF PARTICLES

In practice it is desirable to be able to monitor the field which is being counted, and to be able to see which particles the machine is counting. This is achieved by feeding the pulse that passes to the counter back to the display tube, which brightens the picture as each particle is counted. In this way each particle is effectively marked on the display screen as it is counted (*Figure 5*).

SCANNER FEEDBACK

For consistent counting it is necessary to ensure constant and uniform illumination of the field. This is achieved by sampling the light output from the scanning tube by an additional photocell placed at the eyepiece level. The output of this photocell is amplified by a d.c. amplifier, the output of which is fed back to the scanner tube in a negative feedback loop, thus stabilizing the light output. This results in an improvement in the long term stability of the machine and also an improvement in the signal–noise ratio by reduction in the effects of graininess of the scanner phosphor.

FIELD SELECTION

In the study of sections of biological material, it is often not easy to obtain a uniform area sufficient to fill the microscope field at the magnification required for counting. It is desirable, therefore, to be able to restrict the field counted in both the vertical and horizontal directions, so that only a particular area registers in the counter. Additional pulses, of variable width, derived from the line and frame blanking system, are used to gate the input to the counter, which is therefore only operative over a portion of the line and frame scans. This also enables the field to be divided into small strips from which information as to the distribution of particles within the field can be obtained.

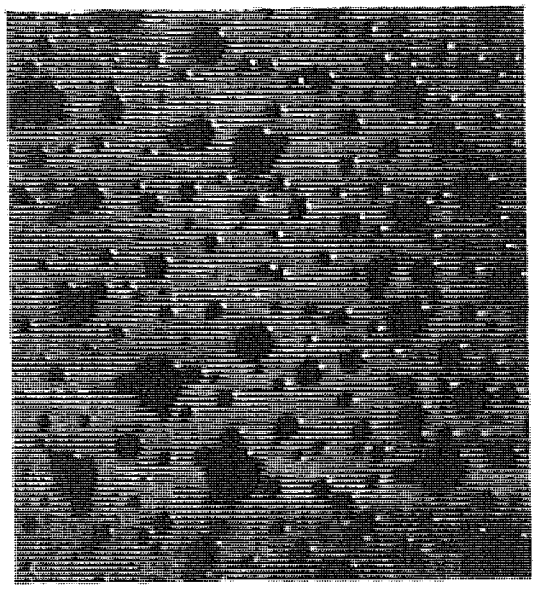

Figure 5. View of test slide with machine presumed to be counting all particles; the bright spots indicate that a pulse is being registered on the counter

The authors' grateful thanks are due to the Nuffield Foundation for its support, and to Messrs Maturana, Michie, Sholl, Stanier and Taylor for their help with counting and otherwise, and to Cinema-Television Ltd for assistance with equipment.

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X-RAY MICROSCOPY

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Three forms of x-ray microscopes—based on the principles of reflection, contact and projection—have been evolved in recent years. Dr Nixon traces their development, and discusses the merits and limitations of each type.

ONE OF THE most recently developed methods of microscopy is the production of magnified images using x-rays. The use of this type of radiation in microscopy has two major and several minor advantages as compared with light or electron illumination. The first advantage is the possibility of achieving a resolution much better than that of the light microscope, although not as great as the electron microscope, due to the short wavelength (1 to 10 Å) of x-rays that are easily absorbed by biological material. At present a resolution of 1000 Å has been attained with x-rays and it is a great encouragement to further development to know that this is only a technical barrier and not the insurmountable Abbé limit reached some time ago by light microscopists.

The second advantage is the different method of contrast formation when soft x-rays are used. These rays are absorbed and not scattered as light and electrons, and the well known x-ray absorption laws can be used to obtain a quantitative chemical picture of the specimen from the x-ray micrograph negative. Both specific element detection and mass determination have already been achieved with x-rays[1]. The following are some further advantages:

(*a*) penetration of thick specimens, enabling internal detail of opaque biological and metallurgical specimens to be seen

(*b*) penetration of the air surrounding a specimen so that neither the specimen nor photographic plate need be placed in a vacuum as in the electron microscope

(*c*) great depth of field, allowing a complete three-dimensional view of the internal detail of a specimen to be seen at one time; this can also be interpreted quantitatively by measuring the amount of motion of the specimen between the two x-ray exposures used to form a stereographic pair

(*d*) the combination of x-ray microscopy and selected area micro x-ray diffraction to produce detailed identification of areas of $1\mu^2$.

All these advantages have stimulated research and discussion on the possibility of producing an x-ray microscope. Refracting lenses directly analogous to the light microscope cannot be used for x-rays since the refractive index of all known materials is almost equal to (but less than) unity *i.e.* about 0·99998. This leads to a focal length some 10,000 times the radius of curvature of the lens and, for a useful magnification, an image distance of up to 100 times the focal length.

x-Ray reflecting mirror systems, also analogous to optical mirrors, have in fact been produced[2] and will be discussed later. The initially predicted diffraction resolution of 70 Å for this method has since been revised to 0·1–0·2μ due to mirror aberrations and imperfections that are most noticeable at the small glancing angles used. A resolution of about 1μ has been demonstrated[2,3].

Most of the advantages listed, with the notable exception of improved resolution, have been obtained with the contact method of microradiography. Some of the first results were achieved less than two years after the discovery of x-rays[4] and modern methods have improved this type of x-ray imaging to make it a generally available routine tool for research and industry[5]. The specimen is placed in close contact with a fine grained photographic plate and exposed to an x-ray beam from a normal tube. All of the enlargement is produced optically and thus the ultimate limit of resolution is equal to that of the light microscope. In practice the granularity or random distribution of the photographic grains and also some fogging by photoelectrons limit the resolution to 0·5μ.

These limitations are avoided in projection microradiography where the specimen and photographic

plate are separated and an initial geometrical magnification of 1000 times is easily obtained, followed by further photographic enlargement. In this way the need for x-ray focusing is obviated. Similarly, as the specimen can be placed closer to the x-ray source, the resolution is not determined by the photographic grain size but rather by the size of the x-ray focal spot. Modern magnetic lenses as used in the electron microscope can produce electron beams well below 1μ in diameter. This type of x-ray microscope has been developed originally at the Cavendish Laboratory to give a resolution of 0·5μ[6–8] and more recently 0·1μ (1000 Å) has been reached[9,10].

REFLECTION X-RAY MICROSCOPY

The need for x-ray reflecting monochromators for x-ray diffraction purposes produced several studies of x-ray mirrors by F. JENTZSCH[11], J. W. M. DU MOND and H. A. KIRKPATRICK[12], Y. CAUCHOIS[13], W. EHRENBERG[14] and others. The possibility of producing magnified x-ray images was noted in some of these earlier papers but not fully developed until P. KIRKPATRICK and A. V. BAEZ designed a two-mirror x-ray microscope with a resolution of 1μ over a small field[2,15].

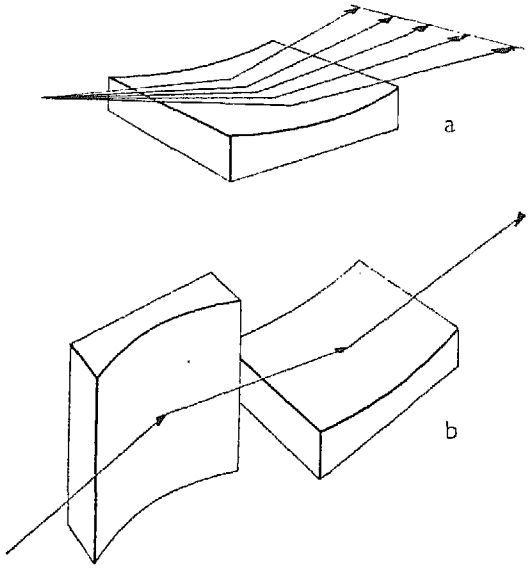

Figure 1. Basic two-mirror element of the reflection X-ray microscope showing rays reflected at grazing incidence: a *a single cylindrical mirror produces a line image of a point object;* b *a second mirror corrects this astigmatism by producing a point image of a line object—the image formed by the first mirror (after Kirkpatrick*[16]*)*

Resolution—The basic focusing element of this type of microscope is shown in *Figure 1*. Since the refractive index for x-rays is close to but less than unity, the critical angle for total external reflection is close to 90° and depends on the wavelength used. The complement of this angle is usually less than 30′ of arc and reaches a maximum of 4° for platinum reflecting x-rays of 10 Å wavelength. The theoretical resolution of 70 Å is found by using this angle α in the usual diffraction formula

$$d=\lambda/2\alpha \qquad \ldots.(1)$$

where d denotes the resolved distance, λ the x-ray wavelength and α the semi-angular aperture. The angle α varies directly with the x-ray wavelength over the range used, so that the resolution for diffraction only is independent of the x-ray wavelength.

Geometrical aberrations—A point source of x-rays is imaged by a single mirror at this small angle into a line image as shown in *Figure 1*a. A second mirror placed perpendicularly to the first will focus this line image back into a point and so correct this type of astigmatism (*Figure 1*b). However, the two mirrors are not at the same axial position, and so the magnification will not be uniform over the field of view. M. MONTEL[17–19] has developed a mirror block of two orthogonal spherical mirrors to correct this aberration and has produced a resolution of 1μ.

Other aberrations are more serious since they limit the field of view. Coma, spherical aberration

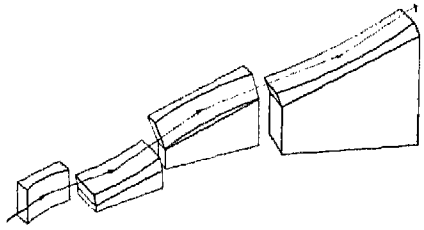

Figure 2. Four-mirror reflection X-ray microscope: the second pair of mirrors corrects the coma and obliquity of the first pair and in addition provides most of the magnification (after Kirkpatrick and Pattee[21]*)*

and distortion are all present, except at the one central point of the field. E. PRINCE[20] has predicted a two-mirror resolution of only 0·34μ by combining the diffraction and aberration limits.

P. KIRKPATRICK and H. H. PATTEE[21] have proposed a four-mirror system, shown in *Figure 2*, where the aberrations introduced by the first two mirrors are eliminated by the second pair. Complete correction over a useful field requires precise figuring of the

mirror surfaces to some, as yet unknown, ideal curve. Ray tracing, using an electronic calculator[22], may help to determine the correct surface before the formidable difficulties of producing such a surface are faced. Even so, some sacrifice of the 70 Å resolution at the centre point of the field must be made to obtain a moderate resolution over a useful area.

A four-mirror compound x-ray microscope, using spherical mirrors and constructed by Kirkpatrick and Pattee, is shown in *Figure 3*. The cover is removed and placed at the left and a 15 cm scale is shown in the foreground together with a sample mirror block. The two micrometers at the top are used to translate the specimen, and the first mirror —hidden near the top—is controlled for tilt by the second micrometer on the right. The second, third, and fourth mirrors are seen, as well as their micrometer controls. When corrected mirrors are produced they will be fitted to this type of mirror block.

A detailed theoretical study of both two-and four-mirror systems has been made by J. DYSON[23] and his estimate is a best resolution of 800 Å for a field of 37·5μ in a four-mirror system. He also points out that this is not much improvement on the simple two-mirror system which should give 1500 Å resolution allowing for aberrations.

Surface aberrations—These geometrical aberrations are all present for perfect reflectors *i.e.* surfaces uniform at every point. Surface finish is equally important as shown by C. M. LUCHT and D. HARKER[3], who have produced a two-mirror x-ray microscope with a resolution of 1μ by using mechanical elastic deformation of optical flats to avoid surface figuring. Electron micrograph replica techniques were used to study the surfaces of the deformed optical flats. A surface smooth to 500 Å was needed for 1μ resolution and a finish to 1000 Å gave noticeably poorer results. There is a factor of twenty between surface finish and resolution here and so a resolution of 1000 Å may require surfaces smooth to 50 Å. W. EHRENBERG[14,24] has also noted the effect of surface finish and striations as opposed to surface shape on x-ray images.

These two limiting factors on the reflection x-ray microscope, surface shape and surface finish, are being studied in detail in Professor P. Kirkpatrick's laboratory at Stanford University, California. Here again the absence of a true diffraction limit on the actual resolution so far achieved encourages a vigorous attack on the technical problems yet to be solved.

Crystal reflection as opposed to mirror reflection has also been tried[25–27], but long exposure times are needed. All types of x-ray reflection methods have been surveyed by H. WILSDORF[28] and H. WOLTER[29], both for x-ray monochromators and true image formation.

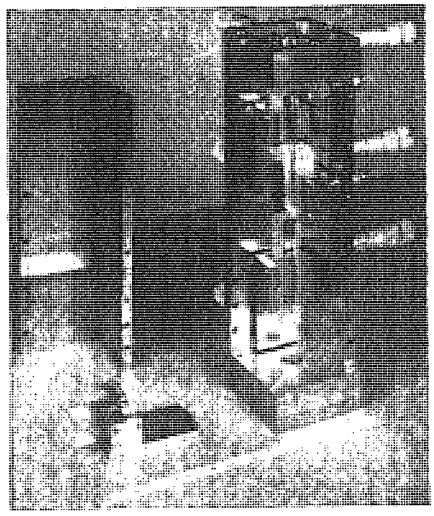

Figure 3. Four-mirror block for reflection X-ray microscope: cover on the left with one mirror; 15 cm scale in front; the top two micrometers translate the specimen; the other four micrometers control the tilt of the mirrors for focusing (after Kirkpatrick and Pattee[21])

CONTACT X-RAY MICROSCOPY

Method—Focusing of x-rays was considered impossible by W. C. ROENTGEN[30] and, as shown above, the successful demonstration of an x-ray microscope with moderate resolution is a fairly recent development. The advantages of x-radiation for microscopy were readily recognized and, despite the lack of an x-ray microscope, F. H. NEVILLE produced 'microskiagrams of a sodium–gold alloy' within two years of Roentgen's discovery of x-rays[4,31].

In this method, now called 'contact microradiography', the specimen is placed in close contact with a fine grained photographic plate and this unit is exposed to x-rays from a normal tube operated at 10 to 20 kv. This x-ray image obtained at unit magnification is subsequently enlarged by an optical microscope. The ease and simplicity of this method are counterbalanced by low resolution and the need for thin specimens.

Resolution—The resolution is impaired by several factors apart from the ultimate limitations of the optical microscope used to magnify the image. The first limit is geometrical blurring due to the

finite size of the x-ray source. The maximum tolerable specimen thickness and minimum distance from the source are both related to the effective size of the source. For example, a specimen 0·1 mm thick and situated at a distance of 10 cm from a 1 mm² source will have a blurring of 1μ. Thicker specimens require a proportionately larger distance from x-ray source to specimen and consequently a longer exposure time due to loss of intensity by the inverse square relation. This is particularly inconvenient with the low voltage soft x-rays used for microradiography where the initial output of x-rays is low and the air absorption is high. A special tube[5], which is available commercially, with a highly loaded focal spot of 0·3 mm square partially overcomes this difficulty and allows 1μ geometrical

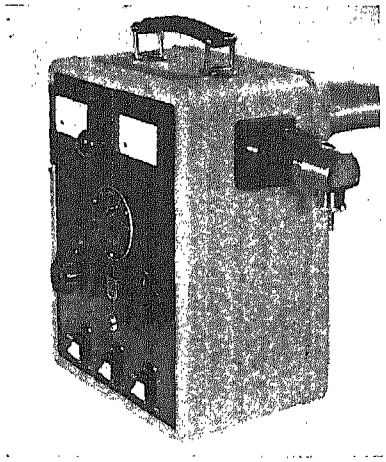

Figure 4. Philips contact microradiographic apparatus: small X-ray tube on right; focal spot=300μ; 1 to 5 kv, 1 to 5 ma, 50μ beryllium window (after Combée, Houtmann and Recourt[5])

blurring for a 50μ thick specimen at 15 mm, giving exposure times of only a few minutes (*Figure 4*).

The grain size and distribution of the silver bromide in the photographic emulsion also limit the resolution. The ' high resolution ' or ' maximum resolution ' emulsions used for microradiography have a grain size as small as 0·1μ but the granularity or random distribution of these small grains is manifested as an unevenness of five to ten times the grain size. This limits the total enlargement to a few hundred times, depending on the specimen. The x-ray image of a test grid or mesh of known size and shape can be magnified 500 times or more and the granularity will not be obtrusive, as shown in *Figure 5*. The ideal black and white contrast of such an object, as well as its thinness, also helps to produce an x-ray image capable of high magnification. An actual specimen of unknown composition such as *Figure 6* cannot be magnified to the same amount and retain the same image quality.

If sufficiently sensitive photographic emulsions can be produced with a grain size of 100 to 200Å, this

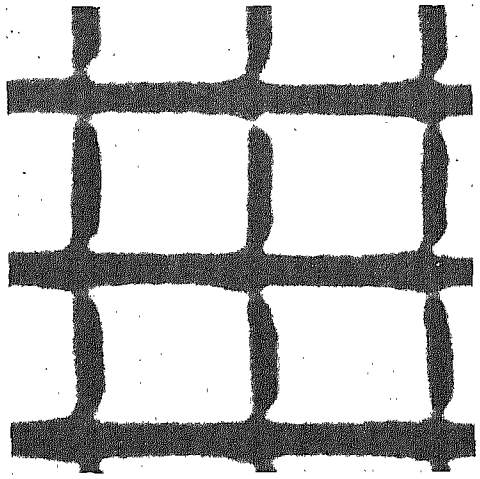

Figure 5. Contact microradiograph using tube of Figure 4: silver grid, 3μ bar width, 3 kv, 3 ma, magnification 1200× (after Combée and Engström[43])

limit will be overcome, allowing a closer approach to the limit of the light microscope. However, in this case the range in the emulsion of the photoelectrons ejected by the x-rays may become the limiting factor. These photoelectrons will be

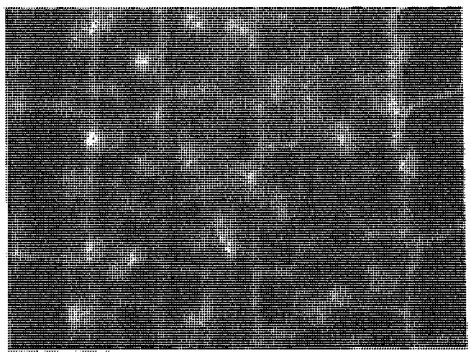

Figure 6. Contact microradiograph using tube of Figure 4: root of Indian corn (Zea Mays)—various cells visible; 10μ section, 3 kv, 3 ma, magnification 280× (after Combée)

directed at 90° to the x-ray path and thereby produce the maximum effect in the emulsion. The energy involved could be as high as the original energy of the electrons in the x-ray tube. At 7 kv the electron range in silver is about 0·25μ, giving a disk of confusion of 0·5μ diameter about any x-ray path, and a larger loss of resolution if the electron path is partially in the less dense gelatin between the grains.

A final limit on resolution is the presence of Fresnel diffraction of the x-ray waves at the specimen and interference at the plate. A similar relation applies as for reflection x-ray microscopy where $d=\lambda/2\alpha$. In contact x-ray microscopy the angle of diffraction at a specimen of thickness b for a resolution d at the plate is $2\alpha=d/b$. Combining these two equations, the resolution is given by

$$d=(b\lambda)^{\frac{1}{2}} \quad \ldots.(2)$$

For a 50μ specimen and 10 Å wavelength x-rays this limit is close to 0·2μ, well below the other limits on contact microradiography at present. However, if a very thin grainless film—sensitive to x-rays and exposed at 1 kv—could be magnified by the electron microscope, this diffraction limit would definitely apply.

Results—All these limits of resolution are below 1μ and a great deal of information has been obtained by contact microradiography down to this level. Metallurgical and biological applications started with F. H. NEVILLE[4,31] in 1897 and P. GOBY[32–34] in 1913, and have gradually been extended as better photographic emulsions became available. A. DAUVILLIER[35] and P. LAMARQUE[36] continued with biological uses, and more recently A. ENGSTRÖM[1,37] has made many improvements in the technique and interpretation of microradiography, particularly of bone sections. Medical research in Sweden has benefited greatly from the pioneering work of Engstrom and its subsequent use by many other medical workers of that country. More complete lists of references are given by A. ENGSTRÖM[1,38] This type of quantitative contact microradiography is also being taken up in Holland[5] and the United States[39–41].

The development of quantitative analysis of the dry weight of a specimen and specific element detection will be discussed in more detail as it can be applied with any method of x-ray microscopy, with a resolution equal to the limit of detection of the x-ray microscope used; it may be used for biological or non-biological specimens. The original method[1] compares the x-ray photographic density of a specimen of known thickness with that of a step wedge of similar material by using a microdensitometer[42]. The wedge is usually made of strips of collodion to match the carbon, nitrogen and oxygen of most biological materials. The absolute value of the mass of any small area is then found from the known mass absorption coefficients of carbon, nitrogen and oxygen. Various correction factors are needed to allow for the spread of x-ray wavelengths used and the different coefficients for these elements at various wavelengths. The final result will give the equivalent dry mass of a volume as small as $1\mu^3$ or less than 10^{-12} gm.

More recently[43] it has been possible to dispense with the reference step wedge of collodion and instead to take several different exposures of the same sample on one film. In this way the film is calibrated for density *versus* exposure and the absolute mass determined by comparing the x-ray absorption with that of a known distance of air.

Engström has also succeeded in detecting specific elements in biological tissue. Two x-ray micrographs are obtained, using x-rays from both sides of the absorption edge of the element sought. The wavelength can be varied by using different x-ray targets to obtain characteristic radiation from copper, cobalt, manganese, iron *etc*, or by varying the voltage in an x-ray tube with a single target such as tungsten. The two micrographs are compared and the element to be found will appear darker in one and lighter in the other while the background will change only very slightly. The limit is again determined by the resolution of the method of microscopy used, or about $1\mu^2$ with contact microradiography at present. This type of element detection is also applied in metallurgy, with fluorescent excitation of two characteristic x-ray wavelengths[45], or different x-ray tube targets[46].

G. L. CLARK[47,48] has developed the metallurgical uses of contact microradiography; J. J. TRILLAT[49], S. E. MADDIGAN[50], and W. BETTERIDGE and R. S. SHARPE[46] have also contributed to this subject. Indeed, this branch of contact microradiography has evolved to such an extent since 1945 that no overall view of metallurgical uses is possible in this survey.

Each branch of metallurgy has used microradiography as an addition to more standard techniques and only a partial list of problems will be given here. The following have been studied: segregations and inclusions[46,51,52,53], castings[54], cast iron[55], diffusion[56–58], deformation[59,60], grain boundaries[61,62], magnets[63], welding[64,65], strain[66], distortion[67] and, phase formation[68]. General metallurgical reviews by W. BETTERIDGE[69], F. FOURNIER[70] and G. A. HOMÉS and J. GOUZOU[71] list many other references. Other industrial applications are also too numerous to discuss in detail and will only be listed. Ceramics[72], insulators[73], minerals[74], ores[75], plastics[76], paper[77],

paints[78], cloth[79], and rubber[80] have all been investigated.

Contact microradiography can easily be adapted to stereoscopic viewing by tilting the specimen through 10° between two exposures. The thickness limitations mentioned earlier still hold and so a resolution of 1μ will only be found in a stereoscopic view of a specimen less than 50μ thick. At 500 times enlargement this specimen would appear about 1 in. thick in a stereoscopic viewer. Thicker specimens can also be used but only with some loss of resolution.

P. GOBY, who coined the word 'microradiography'[32,81], also demonstrated the first stereographic x-ray micrographs[82]. Other stereographic work has been done by U. YOSHIDA and H. TANAKA[83], H. F. SHERWOOD[84], G. L. CLARK and R. W. EYLER[85], R. BERTHOLD[86], S. BELLMAN[87], and W. M. WILLIAMS and C. S. SMITH[62]. The full value of seeing the internal detail of a specimen can be appreciated by viewing any of the best results such as the beautiful stereographs of aluminium–tin alloys of Williams and Smith. The qualitative aspect of three-dimensional viewing can also be placed on a quantitative basis by similar methods to those used in aerial photography and electron microscopy[88]. The angle of tilt and measurements on the two micrographs will give the exact separation in depth of any two visible features of the specimen.

Most of the results listed here have been obtained with normal x-ray tubes and equipment modified for low voltage microradiography. The recent introduction of a special x-ray tube designed for the contact method[5] will probably bring about a great increase of activity in this field (with a resolution of 1μ).

PROJECTION X-RAY MICROSCOPY

Method—The geometrical blurring of a contact microradiograph can be reduced by using a smaller x-ray source, as noted earlier. In projection x-ray microscopy a point source of x-rays is used and much thicker specimens can be examined. In fact the specimen does not need to be in contact with the photographic plate and by moving it closer to the x-ray source a certain amount of geometrical x-ray enlargement is possible. This 'shadow projection' method of image formation shifts the chief limitation on resolution from the granularity of the film to the size of the x-ray source.

This method of microscopy was suggested by R. SIEVERT[89] in 1936 and M. VON ARDENNE[90–91] in 1939 and others, and subsequently developed at Cambridge[6–8]. The basic principle of the special x-ray tube needed is shown in *Figure 7*. A normal triode-type of electron gun produces an electron beam at 5 to 20 kv which is reduced in size by two magnetic electron lenses. This beam strikes the thin metal foil target and produces an x-ray source of 0·1 to 1μ in the vacuum wall of the apparatus. In this way the electron path is in vacuum but the x-ray path can be at atmospheric pressure. An object placed

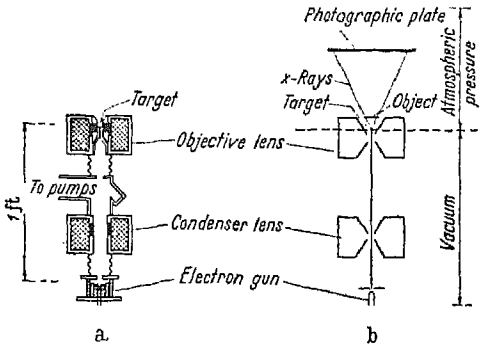

Figure 7. Projection X-ray microscope: a *cross-section of instrument;* b *schematic diagram (after Cosslett and Nixon)*

close to this point source of x-rays casts an enlarged shadow image on to a fluorescent screen or photographic plate some distance away. The initial x-ray magnification is given by the ratio of source–plate distance to source–object distance and the resolution is limited in the first place to the size of the x-ray source. In practice x-ray magnifications of 1000 times have been used (plate at 10 cm, object at 0·1 mm) with further photographic enlargement, and a resolution of 0·1 to 1μ depending on voltage, target and specimen.

Resolution—The limits on resolution with this method are similar to those discussed for reflection and contact x-ray microscopy. The ultimate limit is given by Fresnel diffraction of the x-ray waves and equation 2 also holds here. In this case b is the distance of the specimen from the x-ray source and not the distance from the photographic plate. For a wavelength of 5 Å, and a resolution of 1μ, $b=2$ mm. This small object distance is easily obtained with an x-ray tube of the end window type shown in *Figure 7*. A normal type of solid anode x-ray tube could not be used for x-ray microscopy, even with a point source of x-rays, because of the geometrical restrictions on a small object distance.

For a resolution of 0·1μ, b falls to 20μ and this also can be obtained with an end window tube since the target is only 1 to 3μ thick. Here again it would be impossible to have an object in air only 20μ from the x-ray source of a normal tube. Fortunately this

small object distance automatically gives a high x-ray enlargement with a short plate distance and short exposure times even at 0·1μ resolution.

The smallest useful size of the x-ray source is limited by the intensity available in the electron beam. Electron lenses are completely uncorrected for spherical aberration and a semi-angular aperture of 10^{-2} rad. must be used compared to the large numerical aperture of a glass objective, which may be as high as 1·5. In an electron microscope using such a small angular aperture, exposure times of a few seconds are still possible due to the enormous brightness of the electron source. In the x-ray projection microscope, however, the conversion efficiency of electrons into x-rays is very poor—about 0·1 per cent, the rest of the electron power appearing as heat. This means that 5 min. exposures are necessary with an electron source of 1000 Å, and considerably longer ones if an electron source of 100 Å were used. It is interesting to note with V. E. COSSLETT[92] that the intensity limitation is given by the brightness of the electron source and not by the power dissipation of the metal foil target. No water cooling or rotating anode is needed here since the total load is at most 1w (50μa at 20 kv).

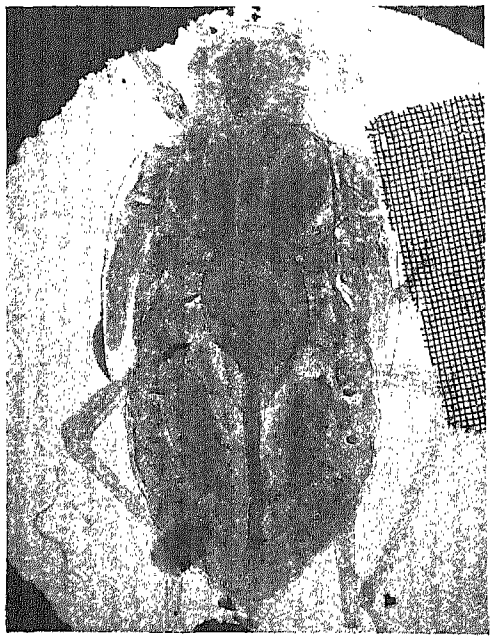

Figure 8. Projection X-ray micrograph (10 kv, 5 min. exposure): aphid, Aphis fabae, *freeze dried; X-ray magnification 20×, final magnification 40×,1500 mesh/in. silver grid, 3μ bars (after Cosslett and Nixon[8])*

A final limitation on the size of x-ray source is the penetration and scattering of electrons within the foil target. At 10 kv the penetration produces x-rays from a hemisphere of 0·5μ diameter and this forms the present limit of resolution unless the kilovoltage is lowered or the target is thinner than the electron range.

Results—The projection x-ray microscope has been used with a variety of specimens in order to test resolution and performance. An x-ray micrograph from the biological field is shown in *Figure 8*. The specimen is a freeze dried green fly or aphid, *Aphis fabae*, together with a fine mesh silver grid of 3μ bar width. The internal detail of the wing buds and developing ovaries can be seen, as well as the coiled digestive tract. The resolution here is about 0·5μ.

A metallurgical example is shown in *Figure 9*. This is nodular cast iron, 3 per cent carbon by weight,

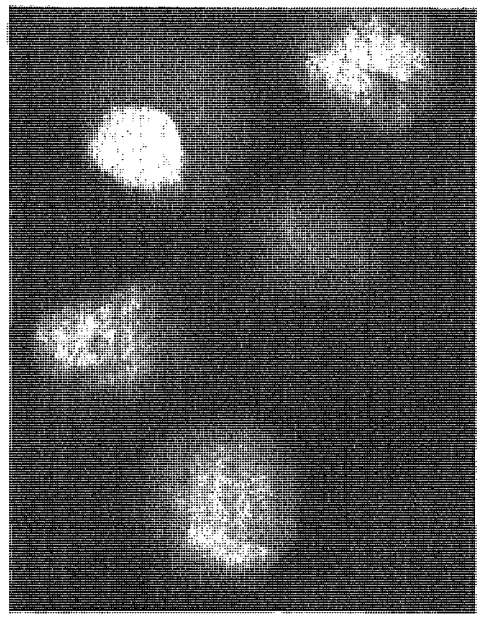

Figure 9. Projection X-ray micrograph (20 kv, 5 min. exposure): nodular cast iron—3 per cent carbon; magnification 700× (after Nixon and Cosslett[102])

and shows the graphite spheres as light areas. The specimen is 75μ thick and the spheroids are about 25μ in diameter. A stereoscopic view, not shown here, gives the true shape and distribution of the nodules within the iron. This magnetic specimen can be used with the magnetic lens x-ray microscope since the small specimen saturates in the magnetic

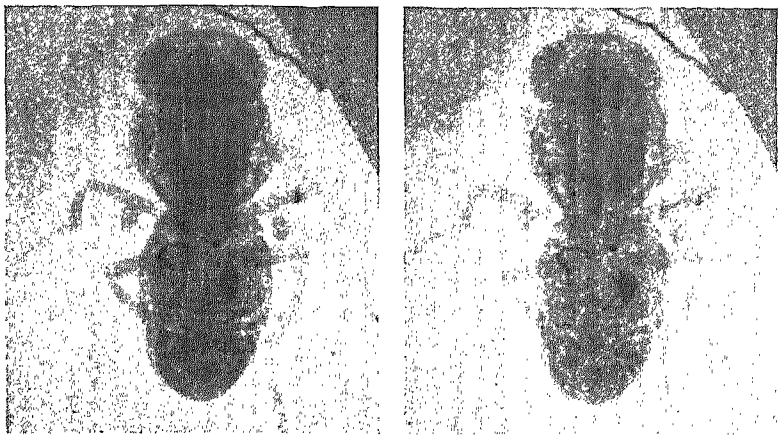

Figure 10. Projection X-ray micrograph (10 kv, 5 min. exposures): stereograph of a fruit fly, Drosophila melanogaster *and 1500 mesh/in. silver grid (after Cosslett and Nixon[7])*

field and causes very little disturbance to the focusing of the electrons. It is only necessary to hold the specimen securely during the exposure.

A stereoscopic view of a fruit fly, *Drosophila melanogaster* is seen in *Figure 10.* A small hand viewer will help in merging the two images to form one three-dimensional view. The legs of the insect come out from the plane of the paper and the piece of 1500 mesh grid is in the background.

The reduction of geometrical blurring with projection x-ray microscopy means that the whole of the specimen is in focus at once, with the different planes magnified by different amounts. Because of this large depth of field, stereographic results can be obtained even at the highest magnification. Tilting the specimen at low magnification between two exposures or traversing the specimen across the cone of x-ray illumination at high magnification will both produce stereographic views. This combination of viewing the internal detail of a specimen, all in focus at once, and in three dimensions is one of the main advantages of this method even when the resolution is no better than that of the light microscope.

Commercial models—The many advantages of projection x-ray microscopy have led to commercial development of this instrument in at least three countries. The first published prototype[93]—made in the United States and stemming directly from the developments at the Cavendish Laboratory, Cambridge—is shown in *Figure 11.* This instrument was designed with electrostatic lenses by W. C. NIXON and S. P. NEWBERRY[94] to give an inexpensive model that would have to sacrifice some resolution and intensity to the more powerful magnetic lens x-ray microscope. In actual use the resolution is about 1μ, limited by electron penetration in the target, with exposure times of 20 min. This is a continuously pumped x-ray tube with a diffusion pump within the base and an external backing pump not shown. The two cylindrical vacuum valves are operated by levers on the left of the large square camera. The three meters are for vacuum, beam current with overload cut-out and high voltage.

At present this instrument has only a few limited applications where an initial geometrical x-ray enlargement is needed since the simple tube of *Figure 4* has equal resolution and less exposure time at a far lower cost. The advantages will swing the other way for the magnetic lens x-ray microscopes, both pumped and sealed off tubes, now being developed.

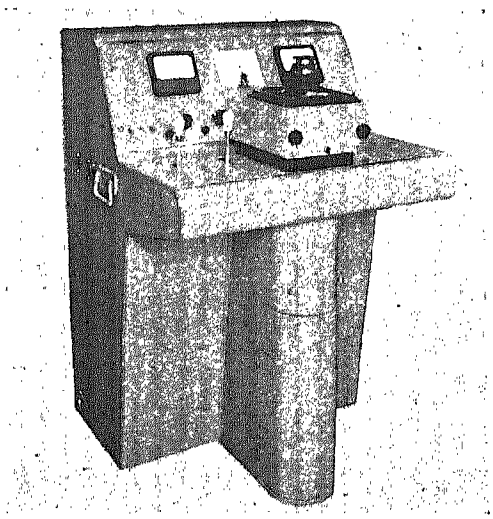

Figure 11. General Electric projection X-ray microscope (resolution 1μ, exposure time 20 min.): electrostatic lenses (after Newberry and Summers[93])

High resolution—Recent developments have produced a resolution of 0·1μ (1000 Å)[9,10] with the projection x-ray microscope and a test grid is shown in *Figure 12*. This is the same type of grid as seen in *Figure 5* and with the aphid in *Figure 8*, but it is magnified to 2000 times. Most of the grid bars are outlined by a faint white fringe due to Fresnel diffraction of the x-ray waves. At some places this fringe is 0·1μ in width, indicating a resolving power of this order. Gold shadowed bull sperms, having tails 0·3μ across and latex particles of 0·25μ diameter,

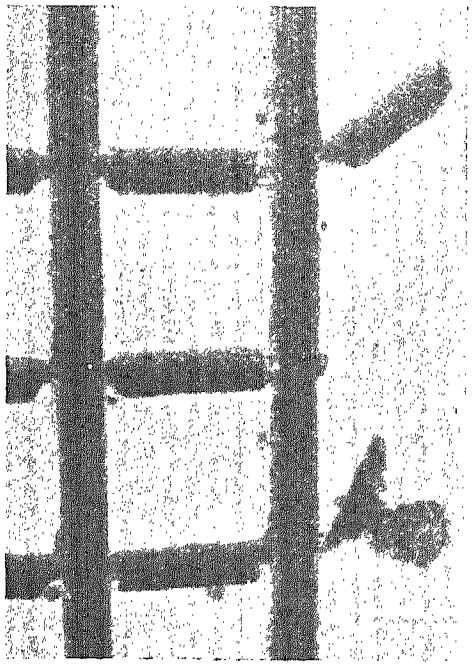

Figure 12. Projection X-ray micrograph (10 kv, 5 min. exposure, 1500 mesh/in. silver grid, 3μ bars, magnification 2000×): same grid as Figures 5 and 8; Fresnel fringe (white) around grid 0·1μ wide (after Nixon[9])

have also been imaged to show a resolution similar to that given by the fringe width.

The smaller x-ray source has been produced by using 0·1μ gold leaf as an x-ray target. This foil is semi-transparent to 10 kv electrons and eliminates some of the enlargement of the source due to electron scattering in thicker targets. It was found to be impossible to avoid this enlargement of the x-ray source at 10 kv by using a thin foil backed with a thicker beryllium layer, since an appreciable amount of x-rays is generated in the beryllium which spoils the resolution. The two solutions are to use thinner targets (as has been done) or to reduce the kilovoltage, which is difficult with the present electron guns since the intensity also falls rapidly.

CONTRAST LIMITATIONS

Each of the three forms of x-ray microscopy discussed has certain merits for image formation. However, all three are equally concerned with the problem of contrast in the x-ray image, both for present performance and future improvements in resolution.

J. L. FARRANT[95] has pointed out the need for long wavelengths with any x-ray microscope when using biological specimens owing to the low absorption. His half-value layer for cellulose using Cu$K\alpha$ at 1·54 Å is 700μ. This falls to 5μ for Al$K\alpha$ (8·3 Å) and 1·4μ for C$K\alpha$ (44·5 Å). In practice a 50 per cent absorption of the x-rays is not necessary and less than 5 per cent can easily be seen. Since the absorption follows an exponential law, the corresponding thickness of specimen falls rapidly. The thicknesses of carbon, copper, silver and gold for 5 and 1 per cent absorption at 3·6 and 8·3 Å are shown in *Table I.*

Table I. Absorption of X-Rays by Various Substances: Thickness (Å) Giving 5 per cent and 1 per cent Absorption

Substance	$\lambda = 3{\cdot}6$ Å		$\lambda = 8{\cdot}3$ Å	
	5%	1%	5%	1%
Carbon	41,000	8,100	3,500	685
Copper	1,160	230	167	33
Silver	360	70	270	53
Gold	197	39	170	33

The two percentage absorption figures represent the contrast limits from easily seen to possibly not visible. The value of 3·6 Å is chosen as a mean wavelength for a projection microscope using a thin end window at 10 kv. The 8·3 Å value is the Al$K\alpha$ emission line and is produced by using an aluminium target in the x-ray tube in any of the three methods. J. F. MCGEE[96] has described a long wavelength reflection x-ray microscope, and W. EHRENBERG and W. E. SPEAR[97] have used a monochromator to select long wavelengths for contact x-ray microscopy from a normal tube (special window) run at high voltage.

Carbon represents the type of absorption from dried but otherwise untreated biological tissue. A resolution of 0·1μ would mean only 2 per cent contrast from details of the same thickness and even this would require Al$K\alpha$ radiation. Thus, high resolution x-ray microscopy of untreated biological specimens is hardly possible. However, the high absorption of copper for Al$K\alpha$, due to the copper *L* edge at 13 Å, may indicate the chance of observing

naturally occurring elements such as zinc if present in sufficient quantity. These values for copper also show the absorption of the group of elements close to iron and thereby the contrast expected from some metallurgical specimens.

The absorption of silver indicates that the use of heavy metal stains, such as silver for nerve fibres, may be the best means of obtaining contrast at high resolution. Gold does not give much improvement over silver but could be used as a shadowing material as in electron microscopy. This has been done for bull sperm and latex particles, both of which are otherwise invisible at 10 kv.

MICRO-DIFFRACTION

No details of x-ray diffraction will be given in this survey but the general method should be mentioned[98]. The projection x-ray microscope can equally well be called ' a point source x-ray tube for x-ray diffraction '. By using a copper foil target a very bright $1\mu^2$ x-ray source of Cu$K\alpha$ can be produced and a suitable aperture or collimator will give an intense beam of x-rays of this size. The specimen and film can be placed very close to the x-ray source because of the end window. A total x-ray load of 1w is the same as a specific load of 1000 kw/mm^2 or some 1000 times that on the target of a normal tube. Exposure times are much shorter and the resolution is as good as in any micro-beam diffraction method. In addition it is possible to obtain x-ray diffraction patterns from selected areas and to combine the x-ray micrograph and diffraction pattern from a few square microns of the specimen. This is analogous to selected area electron diffraction using the electron microscope. In this way quantitative information can be obtained about the composition of small areas of any x-ray micrograph being studied. Similar x-ray diffraction studies may be made with the reflection x-ray microscope as suggested[21] by Kirkpatrick and Pattee. In their scheme the microscope mirrors reduce the beam of x-rays to a small area of the specimen.

FUTURE DEVELOPMENTS

Further improvement in resolution should be possible for both projection and reflection x-ray microscopy and depends partially on the x-ray intensity available. The use of an x-ray image intensifier as employed in medical fluoroscopy[99], if adapted for low voltages, would permit smaller x-ray sources for projection microscopy and smaller angular apertures for reflection microscopy. This would make possible an improvement in resolution for both methods.

The contrast would be available with heavy metal staining as shown earlier. The diffraction limit would still exist but the presence of a Fresnel fringe as illustrated in *Figure 12* may even help to show the outline of any density differences in a transparent specimen. Phase contrast is not possible in the x-ray microscope since images are formed by absorption and not scattering, so that the two components of illumination cannot be separated.

If the diffraction limit is reached, the two-wavelength reconstruction microscope of D. GABOR adapted to x-rays as suggested by A. V. BAEZ[100] might be used to provide clear images. Alternatively the scanning (flying spot) x-ray microscope of H. H. PATTEE[101] offers several advantages for possible improvement at the cost of greater complexity of associated equipment.

CONCLUSIONS

The three main methods of modern x-ray microscopy have been summarized to show that, although the resolution at present is only equal to that of the ultraviolet microscope, a large number of applications have already been found. Discussion of the various limits on resolution has revealed that further improvements are to be expected, while still retaining the many advantages peculiar to x-ray illumination. Sufficient contrast will be available from metallurgical and stained biological specimens even at the highest resolution expected.

All three types of x-ray microscopes should be used more extensively in the future as the many advantages of the method become known. These instruments are still in many ways experimental models and a certain time lag will occur before sufficient engineering development can place simple and inexpensive x-ray microscopes in all interested laboratories. Yet it is only with widespread use of this new technique by competent workers in all fields that its great potentialities as a scientific tool will be realized. A light microscope serves as the symbol of *Research* for good reasons; the electron microscope represents the new power of investigation at the atomic and molecular level. It may be hoped that the x-ray microscope will join these two as an equally indispensible instrument for providing the detailed knowledge necessary for the continual advancement of science.

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THE POLARIZING MICROSCOPE IN ORGANIC CHEMISTRY AND BIOLOGY

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This article indicates the quantitative nature of the help which the use of a well equipped polarizing microscope can give to the organic chemist and biologist.

THE MICROSCOPE

WHEN the principal users of the microscope were the amateur investigators of natural history no microscope was considered complete unless it had auxiliary apparatus for the production of polarized light. The use of simple polarizing apparatus in the microscope without the complications of the petrological microscope is capable of yielding a great deal of information about the structure of biological material even without staining. In many cases polarized light gives a power to adjust contrast in a specimen, which is very valuable in investigations of structure.

LIGHT

Light is a form of radiant energy having the dual character of an electromagnetic wave and of a particle behaving as if it had the characteristics of wave motion. For the purposes of this essay light will be considered only as an electromagnetic wave form of energy.

Isotropic substances are those in which light travels in every direction with the same velocity. Anisotropic substances are those within which the velocity of transmitted light varies with the direction of propagation. Crystalline materials belonging to the cubic or isometric system or unstrained non-crystalline solids, are isotropic; all crystalline substances belonging to the other crystal systems and non-crystalline solids in a state of strain are anisotropic.

In isotropic substances monochromatic light, that is light of a single wavelength, generated at a point within the substance travels outwards with constant velocity along radial lines. The surface formed by the extremities of these radial lines after a given interval of time is called the ray velocity surface or wave surface. In an isotropic medium the ray velocity surface is a sphere. The ray velocity surface or wave surface in an anisotropic medium may assume almost any shape which can be formed as the surface of a solid of revolution, the limits being a plane and a sphere.

The wave front is a plane tangential to the wave surface at the point of intersection of the surface and of a ray. In isotropic substances it is always normal to the ray. The wave normal is perpendicular to the wave front and in isotropic substances coincides with the direction of the ray. *Figures 1–8* show the properties of waves of importance to the microscopist. *Figures 1* and *2* show the ray velocity surfaces for isotropic and anisotropic substances respectively; in each figure *OR* represents a ray originating at a point source *O*. During a given interval of time light originating at *O* travels along *OR* and reaches *R* on the ray velocity surface. *FF'* gives the position of the wave front, and *RN* that of the wave normal, which in the case of an anisotropic substance does not coincide with the direction of the ray.

WAVE MOTION

Wave motion in its simplest form consists of a combination of uniform forward motion with simple harmonic oscillation at right angles to the direction of forward motion. Consider *Figure 3*: a point rotates about the circle in the direction of the

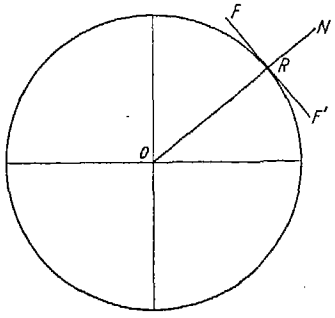

Figure 1. Ray velocity surface for an isotropic substance (after Wahlstrom[1])

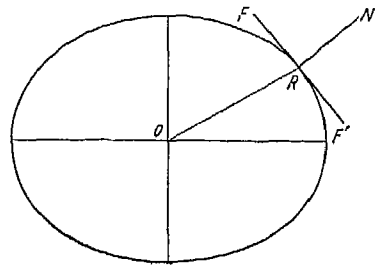

Figure 2. Ray velocity surface for an anisotropic substance (after Wahlstrom[1])

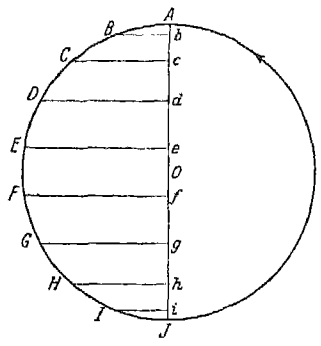

Figure 3. Simple harmonic motion (after Wahlstrom[1])

arrow; at regular time intervals project the position of the point on to the diameter *AJ*. The projections of the positions *A*, *B*, *C*, *D*, *etc* are *A*, *b*, *c*, *d*, *etc*. The oscillatory character of the movements of the projections along *AJ* is simple harmonic motion. The maximum velocity is at the centre *O*, the minimum velocity at *A* and *J*. The amplitude of the wave is *OA* or *OJ*. In light waves intensity is proportional to the square of the amplitude. The period is the time taken to complete a backward and forward motion of the projections of the point, that is the time taken to travel from *A* to *J* and back to *A*. The nature of the wave motion is shown in *Figure 4* in which a wave starts at *O* and moves in the direction *ON*. The wave consists of regular forward motion towards *N* and simple harmonic vibration parallel to *AJ*. The wave is *OKLMN*, *ON* the wavelength, *OA* the amplitude and the time taken to travel from *O* to *N* is the period.

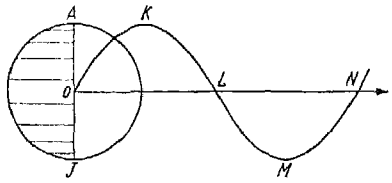

Figure 4. Simple wave motion (after Wahlstrom[1])

INTERFERENCE OF LIGHT WAVES

Light waves from the same source, of the same wavelength, vibrating in the same plane and travelling at the same velocity in the same direction can interfere with each other. *Figure 5* shows the effect of two such waves travelling together, that is, in the same phase. The result is that the amplitudes are added together and the light appears more intense. In *Figure 6* two waves of light have the same wavelength and amplitude but one is half a wavelength ($\lambda/2$) out of phase with the other. The summation of the amplitudes leads to zero amplitude throughout, zero intensity and therefore darkness. *Figure 7* shows the effect of the interference of two waves $\lambda/4$ out of phase, while *Figure 8* shows the effect of the interference of unlike waves producing an irregular wave of varying amplitude and length.

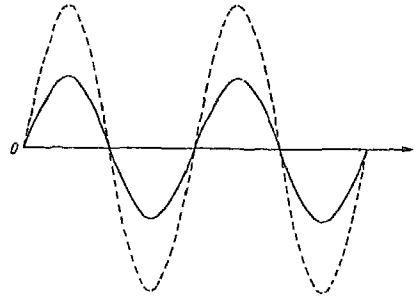

Figure 5. Constructive interference of two waves of equal phase, wavelength and amplitude: resultant, broken line (after Wahlstrom[1])

If two waves vibrate in planes at right angles to each other and are viewed along the direction of transmission several possibilities arise. If the two waves vibrate with phase differences of a whole or a half wavelength then the appearance is that of a straight line; that is, the vibration is along either *AB* or *CD* of *Figure 9*. If the phase difference is one quarter or three quarters of a wavelength, the combination produces the appearance of circular motion. The light is then said to be circularly polarized. If the phase difference is anything except an even or odd multiple of one fourth of a wavelength the motion will be along an ellipse and the light is said to be elliptically polarized.

While light is a complex aggregation of light of different wavelengths which may have different amplitudes and different planes of vibration all combining to produce a very complex motion, it is nevertheless always capable of resolution into its components by a prism. White light can be resolved into two groups of waves vibrating in two mutually perpendicular planes by anisotropic crystals. Light

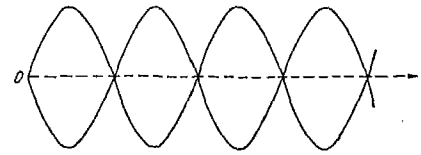

Figure 6. Destructive interference of two waves with the same wavelength and amplitude but with $\frac{1}{2}\lambda$ phase difference (after Wahlstrom[1])

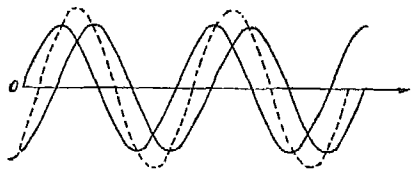

Figure 7. Interference of two similar waves with ½ λ phase difference (after Wahlstrom[1])

vibrating in one plane only is said to be polarized. Unpolarized light vibrates in all planes parallel to the direction of transmission.

POLARIZING MICROSCOPE

Polarized light

The polarizing microscope used by the mineralogist and crystallographer is eminently suitable for use in organic chemistry research and in biological investigation provided certain alterations are made. The polarizing microscope is based upon the ordinary biological microscope with a device—the condenser—for bringing light within the object upon the stage. In optical investigations it will be necessary for the condenser to provide both a parallel beam of light and a convergent beam of light. All the optical fittings which come within the beam of polarized light are required to be free from strain or constituents which might themselves react with the polarized light. A polarizer is situated below the condenser to give a beam of polarized light through the instrument. There are several methods of producing this beam of plane polarized light.

(*i*) *Reflection or refraction*—When light is reflected at a certain angle from the surface of a refracting material, such as glass, light with a vibration azimuth parallel to the plane of the refracting surface tends to be preferentially reflected, whereas light vibrating perpendicularly to the surface tends to be refracted. By selecting either one of these beams and eliminating the other, polarized light vibrating in one plane is obtained. Such a method of obtaining polarized light is not very useful for microscopy although it can occasionally be employed in field work if a normal polarizer is not available.

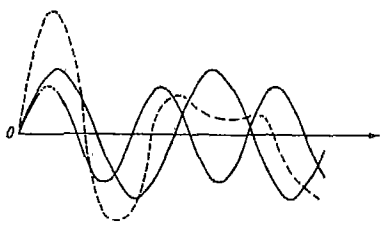

Figure 8. Interference of unlike waves (after Wahlstrom[1])

(*ii*) *Use of a doubly refracting crystal such as calcite*—Calcite can be cut and cemented so that only a beam of polarized light will pass. Calcite has two indices of refraction and can be cut into a prism that causes light with one plane of vibration to be deviated by total reflection while light with a plane of vibration at right angles to the deviated light passes through the prism along the axis of the microscope. A variety of special forms of calcite polarizing prisms have been devised, of which one of the best is the Glan-Thompson. This is preferable to other calcite prisms or to dichroic (absorbing) sheets for critical quantitative work in biology, particularly as an analyser. Calcite prisms are more costly than dichroic polarizers but permit the most accurate reading of extinction angles. The disadvantage is that the aperture is limited by the optical properties of the available calcite.

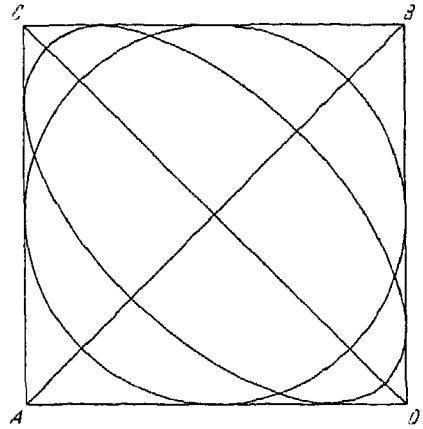

Figure 9

(*iii*) *Absorption in a dichroic medium*—Some natural crystals such as tourmaline have the property of producing polarized light, as have the synthetic *Polaroid* films. *Polaroid* films are less expensive and less bulky than calcite prisms and are not limited by aperture size, permitting the use of ordinary condenser lenses. The disadvantage of *Polaroid* films appears when precise measurements are required. The molecules in synthetic films are not orientated so perfectly as the ions in a good crystal; hence the readings of extinction angles are not so sharp as with good calcite. The films are effective over a smaller colour range and since they absorb efficiently only light with a frequency close to their resonance frequencies other light may be passed. Their efficiency varies with the part of the spectrum being used. A good assessment of the usefulness of calcite and *Polaroid* is given by H. S. BENNETT[2].

The stand

The polarizing microscope stand generally differs from the normal microscope stand in that it has a rotating stage so that the interaction of the object with polarized light may be studied. It will in general perform all the functions of an ordinary microscope with the disadvantage that the tube length is not usually capable of adjustment so that neither correction for cover slip thickness nor the accurate adjustment of eyepiece to objective can be carried out, but it permits the evaluation of properties and characteristics not capable of measurement with an ordinary microscope.

Several variants of the conventional polarizing microscope have been designed to assist the chemist and the biologist in the study of very small objects. It is very difficult to make a rotating stage that will run true for a long time and although there are centring nosepieces and centring adaptors for objectives it is found that small objects tend to rotate out of the field when a high power objective is being used. An ingenious device for overcoming this difficulty was devised by A. B. DICK[3] in which the polarizing prisms were coupled together by a system of gear wheels. The stage thus remained stationary while the optics were rotated round the object. The modern version is an excellent instrument for biological research and for crystallographic examinations in chemical research.

An instrument made specially for the biologist is the M7165 of Cooke, Troughton and Simms. It is an adaptation of their Universal Polarizing Microscope with the $\lambda/20$ mica rotating compensating plate described by A. F. HUGHES and M. M. SWANN[4] and M. M. SWANN and J. M. MITCHISON[5]; it is stated that retardations of $\lambda/1500$ can be measured and $\lambda/2000$ detected. When obtaining one of these instruments the author would advise taking a straight body with an adjustable draw tube. Alternatively a straight tube with a Jackson corrector and sliding objective changers instead of the revolving nosepieces ought to make this an exellent stand for biological work.

MEASUREMENT OF REFRACTIVE INDEX

One of the most generally useful constants which can be measured with the miscroscope is the refractive index. Isometric or cubic crystals have one refractive index, uniaxial crystals have two refractive indices and biaxial crystals three refractive indices. The usual method of measuring refractive index is to place a grain of the material upon a microscope slide with a drop of liquid of known refractive index, near to the expected refractive index of the material. A high power objective is focused upon the grain and then raised slightly out of focus; a white line can then be seen just inside or outside the contact of the grain and the surrounding liquid. This is the Becke line. If the line moves into the grain when the tube is raised the index of the grain is higher than that of the liquid; conversely if the line moves into the liquid when the objective is raised the liquid has the higher refractive index. With care it is possible to find a liquid of the same refractive index as the solid quite rapidly, and in the case of uniaxial and biaxial materials, by altering the orientation of the grain, two or three refractive indices may be measured. The author has found a Jelley refractometer made to the directions given by E. E. JELLEY[6] quite adequate for general work.

THE IDENTIFICATION OF ORGANIC CRYSTALS

In identification of organic crystals a mica $\lambda/4$ and a gypsum first order red plate will be required and should be used just below the analyser.

Preparation of the sample for observation—In the simplest cases preparation of the sample may only involve dissolving in the appropriate solvent and allowing a few drops of the solution to crystallize upon a slide. This is frequently the procedure with inorganic materials. With organic substances, however, the material is often a liquid. In the case of reactive substances, for example amines or acids, simple neutralization with acid or base respectively will in many cases yield a compound of well defined crystalline form. If this product is not sufficiently characteristic, the solution may be treated with a suitable precipitant, and the precipitate examined. Thus treatment with chlorplatinic acid, dipotassium mercury iodide or ferrocyanic acid will usually produce characteristic crystals if the substance is an amine, while treatment with silver nitrate, mercurous nitrate, lead nitrate *etc* will give crystalline precipitates with many organic acids. The choice of derivatives is often influenced by the crystallographic descriptions available. However, if none exist, comparison with a known preparation must be made, and this allows considerable choice. If poor crystals are obtained other solvents should be tried or the rate of evaporation retarded by cooling. Rubbing the edge of the slide with a glass rod is beneficial.

Great care should be taken to use only the purest solvents since traces of oil, resins *etc* in solvents will often delay, deform or completely inhibit the growth of crystals. Small traces of impurities tend to alter the superficial appearance of crystals and in extreme cases even favour a change of habit or crystalline form.

When precipitating a substance upon the slide, it is desirable to choose concentrations of reagents

and sample such that a small amount of precipitate is formed immediately but not so much that the form of the individual crystals is obscured. Spontaneous evaporation of solutions of soluble salts generally yields the best crystals, although gentle heating over a micro-burner is permissible. Rapid evaporation of the solvent may produce deformed crystals.

With the less reactive organic liquids, for example aldehydes, ketones and alcohols, solid derivatives should be prepared according to the usual methods of organic chemistry. If sufficient material is available, it is best to make the preparation on a laboratory scale, thus allowing for losses through purification. If this is not possible, the semi-micro scale may be adopted. When the material tested is sufficiently reactive the preparation may sometimes be carried out on the slide and the excess of reagent removed by alternate washing and decantation with the aid of filter paper. Reagents for aliphatic alcohols and carbonyl derivatives must be selected with care if a solid reaction product is to be obtained. The use of reagents of high molecular weight is valuable for this purpose. Heavily substituted phenylhydrazines are useful for aldehydes and ketones, while 3:5-dinitrobenzoyl chloride is a suitable reagent for alcohols of less than six carbon atoms.

Methods of identification—The identification of a crystalline preparation is accomplished by observing the shape, colour, symmetry, and action of typical crystals on polarized light. The symmetry is not always readily discovered by simple inspection, but may require actual measurements of the crystal angles. Some crystals possess superficial peculiarities of shape which are readily recognized. An investigation by means of purely goniometric methods would, however, require an excessive amount of time and the use of apparatus of more than ordinary precision. Fortunately certain optical properties of crystals associated with the geometric properties are capable of indicating in positive fashion the order of symmetry possible in a given case. Certain of these properties, such as extinction angle (in the case of oblique extinction), birefringence, and pleochroism, may be highly specific.

Crystallographically a substance is classified according to the six systems, *isometric*, *tetragonal*, *hexagonal*, *rhombic*, *monoclinic* and *triclinic*, and their numerous classes. Optically, crystalline substances are either isotropic or anisotropic. Isotropic substances are without action upon the plane of polarized light, and the crystals will remain dark in all positions if viewed through a microscope with crossed nicols. Anisotropic substances under similar circumstances become illuminated and extinguish while rotated through 90°. This phenomenon of alternately becoming light and dark at regular intervals is known as extinction.

There are two types of extinction possible, parallel and oblique. Parallel extinction is found in the tetragonal, hexagonal, and rhombic systems. Oblique extinction is a property of the mono- and triclinic systems. The former system exhibits parallel extinction in certain planes. Parallel extinction occurs when the cross hairs of the ocular are parallel to a principal crystallographic direction (or bounding plane) of a crystal, and in oblique extinction the crystal extinguishes in a position oblique to the particular crystallographic direction. In oblique extinction, the angle between the cross hairs and the position of extinction is constant for a given substance with respect to the particular crystallographic direction. This angle is commonly known as the extinction angle, and is usually taken as the smaller of the two possible angles.

Anisotropic substances are further subdivided into uniaxial and biaxial groups. The former includes the tetragonal and hexagonal systems, while to the latter belong the rhombic, monoclinic and triclinic systems. The distinguishing feature of uniaxial crystals is the exhibition of parallel extinction in two crystallographic directions and isotropy in the third crystallographic direction, known as the optical axis. Biaxial crystals exhibit extinction in all crystallographic directions, although the extinction is not so complete as with uniaxial substances. Two optical axes exist, which, except in the case of rhombic crystals, do not ordinarily correspond to any crystallographic direction. The critical distinguishing feature between uniaxial and biaxial substances is the shape of the interference figure, which will be discussed later. The presence of a lower order of crystallographic symmetry will often disclose the biaxial character of a crystal.

Anisotropic substances have the property of resolving rays of light entering them into component rays vibrating in planes perpendicular to each other. Since the index of refraction of the substance varies in different directions, one ray is retarded with respect to the other. This ray is called the slow component and the other the fast component. These mutually perpendicular rays are further classified according to their relation to the plane of the optical axis or axes. In the case of uniaxial crystals this classification is simple. The component vibrating perpendicular to the optical axis is called the ordinary ray while that vibrating parallel to the axis is the extraordinary ray. The ordinary ray may correspond to the slow component and the extraordinary ray to the fast, or *vice versa*. This serves as an additional

Table I. Newton's Colour Scale (Modified from Quinoke[7])

No.	*Retardation λ=589mμ mμ*	*Order*	*Interference colours between crossed nicols*	*Interference colours between parallel nicols*
1	0	0	*Black*	*Bright white*
2	40		*Iron-grey*	*White*
3	97		*Lavender-grey*	*Yellowish white*
4	158	1/4	*Greyish blue*	*Brownish white*
5	218		*Clearer grey*	*Brownish yellow*
6	234		*Greenish white*	*Brown*
7	259		*Almost pure white*	*Light red*
8	267		*Yellowish white*	*Carmine*
9	275		*Pale straw-yellow*	*Dark reddish brown*
10	281		*Straw-yellow*	*Deep violet*
11	306	1/2	*Light yellow*	*Indigo*
12	332		*Bright yellow*	*Blue*
13	430		*Brownish yellow*	*Greyish blue*
14	505	3/4	*Reddish orange*	*Bluish green*
15	536		*Red*	*Pale green*
16	551		*Deep red*	*Yellowish green*
17	565		*Purple*	*Lighter green*
18	575		*Violet*	*Greenish yellow*
19	589	1	*Indigo*	*Golden yellow*
20	664		*Sky-blue*	*Orange*
21	728		*Greenish blue*	*Brownish orange*
22	747		*Green*	*Light carmine*
23	826		*Lighter green*	*Purplish red*
24	843		*Yellowish green*	*Violet-purple*
25	866		*Greenish yellow*	*Violet*
26	910	3/2	*Pure yellow*	*Indigo*
27	948		*Orange*	*Dark blue*
28	998		*Bright orange-red*	*Greenish blue*
29	1101		*Dark violet-red*	*Green*
30	1128		*Light bluish violet*	*Yellowish green*
31	1151	2	*Indigo*	*Impure yellow*
32	1258		*Greenish blue*	*Flesh coloured*
33	1334		*Sea-green*	*Brownish red*
34	1376		*Brilliant green*	*Violet*
35	1426	5/2	*Greenish yellow*	*Greyish blue*
36	1495		*Flesh-colour*	*Sea-green*
37	1534		*Carmine*	*Green*
38	1621		*Dull purple*	*Dull sea-green*
39	1652		*Violet-grey*	*Yellowish green*
40	1682		*Greyish blue*	*Greenish yellow*
41	1711		*Dull sea-green*	*Yellowish grey*
42	1744	3	*Bluish green*	*Lilac*
43	1811		*Light green*	*Carmine*
44	1927		*Light greenish grey*	*Greyish red*
45	2007		*Whitish grey*	*Bluish grey*
46	2048		*Flesh-red*	*Green*

distinguishing property, and crystals grouped in the first category, where the slow component is perpendicular to the optical axis, are said to possess negative double refraction, and crystals in the latter are said to exhibit positive double refraction. In the case of biaxial crystals where there are two optical axes, the bisectrix of the acute angle between the two optical axes takes the place of the single optical axis of uniaxial crystals in determining the sign of double refraction. Since the acute bisectrix does not necessarily correspond to any crystallographic direction, it is obvious that determining the sign of double refraction of biaxial crystals is more difficult than with uniaxial ones. The procedure for determining the sign of double refraction in uniaxial crystals will be discussed later.

Interference or polarization colours are observed when polarized light passes through a thin section of anisotropic material. The phenomenon is the same as the colour effects observed in soap bubbles and in films of oil spread upon water. It is caused by the retardation of certain wavelengths of light passing through an anisotropic medium and their resulting interference or reinforcement. The colour observed depends upon the thickness of the crystal,

the composition of the light used, and the birefringence (the maximum numerical difference between the several refractive indices of an anisotropic medium), the latter being constant for a given crystalline substance. The observation of brilliant interference colours under crossed nicols should be noted, as it is qualitative evidence of strong double refraction.

When classified according to increasing retardation, the colours fall into recurrent groups called orders, in which the colours become increasingly less saturated. *Table I* is a modification of Newton's classification.

When the thickness of a crystal is known the birefringence—degree of double refraction—may be determined from *Table I* by identifying its polarization colour. The thickness is readily found by focusing the microscope first on the object slide and then on the upper surface of the crystal and reading the displacement from the micrometer screw.

The sign of double refraction of uniaxial crystals is readily determined by rotating the crystal under crossed nicols until the position of extinction is reached. The object is then rotated 45° farther, corresponding to the position of maximum illumination, and the polarization colour noted. The plane of the crystallographic *C*-axis, identical with the optical axis, is determined from the symmetry of the crystal, and a test plate (selenite, first order red, or quarter undulation mica) is placed under the analyser with the vibration direction of its slow component parallel to the optical axis of the crystal. If the resulting interference colour is of higher order than originally, the sign of double refraction is positive, if of lower order the double refraction is negative. If the crystal is rotated 90° from the above position the colour changes are reversed. The test plate producing the more readily interpreted colour change should be used. This is best determined by trial. If the *C*-axis cannot be definitely located, the sign of double refraction cannot be determined by this method. In such a case the sign of elongation may be found. This property is determined with reference to the longest dimension of the crystal, which is not necessarily the direction of the *C*-axis. The procedure is in all other respects the same as that above. The sign of elongation is more arbitrary than the sign of double refraction, but is nevertheless useful.

The optical methods using polarized light described thus far are designated orthoscopic methods, and require only plane or gently convergent polarized light, while the methods that make use of strongly convergent polarized light are called conoscopic methods.

Conoscopic methods—When convergent polarized light passes through a properly oriented anisotropic crystal, a system of interference rings, cut by a dark cross or hyperbolae, may be observed. This image has no connection with the shape of the crystal, but only with the orientation, thickness and optical character of the crystal. Uniaxial crystals presenting a face perpendicular to the plane of the optical axis produce an interference figure consisting of a whole series of alternating light and dark annular bands overlayed by a dark maltese cross. When monochromatic light is employed the dark rings are black; otherwise they are coloured. The number of rings visible depends upon several factors, for example the nature of the light used, the thickness of the section, and the numerical aperture of the lens system.

The uniaxial cross remains stationary during rotation of the section, provided the plane of the section is not changed. If the orientation of the crystal is displaced from the plane perpendicular to the optical axis the cross will become decentred and only a portion of it will remain visible. In petrography, mineral crystal sections can be cut in any desired orientation. In such cases the ability to obtain good interference figures is largely the result of the experimenter's skill in choosing the proper orientations. With microscopic crystals, sectioning and predetermined orientation are not practical. The crystals of some substances ordinarily lie in convenient positions and may be observed directly, but with other compounds the figure may only be obtained with occasional random orientations. Very occasionally a fairly large crystal may be thrown over on the proper face with a fine needle or platinum wire. Where definite cleavage exists crushed crystal fragments sometimes yield good interference figures.

There are certain precautions to be taken in obtaining good interference figures. For example iodoform is crystallized slowly from dilute alcohol solution on an object slide. The crystals are chiefly in the form of thin plate-like hexagons. The microscope objectives must be carefully centred so that the crystal, once located, will remain in the centre of the field during a complete revolution of the stage, or the crystal may pass out of the field of view entirely. When satisfactory centering is assured, a large, well formed crystal is focused and centred using a 4 mm objective. The substage is then slightly lowered and the swing-out condenser above the polarizer placed in the optical system by means of a small lever. The condenser is then raised until it is practically in contact with the object slide. It must not, however, force the slide

against the objective. The crystal is again focused and the eyepiece removed. The analyser is placed over the open tube in the crossed position. Either immediately or upon slight focusing, a small but very clear uniaxial interference figure will be observed. A faint positive uniaxial figure should be entirely disregarded since practically all strong objectives exhibit a faint positive double refraction. The figure produced by iodoform remains perfectly stationary upon revolving the stage.

The method of observing interference figures with the eyepiece removed is known as the Lasaulx method. It is the only method of observation practical with a cap analyser. With a tube analyser a small magnifying lens (Bertrand lens) may be placed in a slot in the tube midway between the analyser and the eyepiece. This arrangement gives larger but less distinct figures than the Lasaulx method. In addition the position of biaxial figures may be observed relative to a cross hair or goniometer eyepiece.

The centre of the dark cross of a uniaxial interference figure, which is also the centre of the concentric interference rings, marks the point of emergence of the optical axis. It appears in the centre of the field only when the observed face of the crystal lies perpendicular to the optical axis. Biaxial crystals possess two optical axes, hence when properly viewed the interference figure presents two points of emergence called melatopes, each surrounded by its system of rings. Instead of the cross characteristic of uniaxial substances, a dark hyperbolic brush extends outward toward the edge of the field from each optical axis. These dark brushes are called isogyres. Contrary to uniaxial figures, biaxial interference figures rotate as the stage is rotated. The shape of the figure is most characteristic when the line joining the melatopes is at an angle of 45° to the cross hairs of the eyepiece. When the line joining the melatopes is horizontal the isogyres unite to form a dark cross similar to the uniaxial figure.

In order to observe a complete biaxial figure the crystal must be lying perpendicular to the acute bisectrix of the optic axial angle, otherwise an incomplete figure is obtained which is more difficult to interpret than in the case of a uniaxial figure. When the crystal is properly oriented, the more of the interference figure can be seen the smaller the optic axial angle. Where this angle is very large, the melatopes and their isogyres may not be visible. In such cases the crystal should be oriented perpendicular to an optical axis and the individual melatope and isogyre examined. With small crystals this orientation of the specimen will be fortuitous unless a universal stage is used. Silver nitrate and boric acid crystals are good examples of biaxial crystals giving good interference figures. Crystallographically boric acid is pseudo-hexagonal. However, the biaxial character of its interference figure is readily apparent, since the unsymmetrical interference cross rotates as the stage moves. Macroscopic crystals absorb too much light, their surfaces are apt to be too badly scarred and are not very satisfactory.

The sign of double refraction may be easily determined from the interference figure by the use of a test plate. A selenite (gypsum) plate, red of the first order, placed over the open tube, just below the analyser, at an angle of 45° to the vibration planes of the nicols, causes the dark cross of a uniaxial interference figure to appear red, while one pair of opposing quadrants becomes blue and the other pair orange. When the vibration direction of the slow component of the test plate is parallel to the line joining the blue quadrants (higher interference colour) the double refraction is positive, and when the slow component of the plate is parallel to the line joining the orange quadrants (lower interference colour) the double refraction is negative. With biaxial crystals the isogyres are coloured red and the space enclosed by the isogyres is orange with the rest of the field blue, or *vice versa*. The rule used above for determining the double refraction is also applied here.

If the quarter undulation mica (Muscovite) plate is used, the dark cross or isogyres disappear and two dark spots are seen. When the spots are aligned perpendicular to the direction of the slow component of the test plate the double refraction is positive. When the spots are aligned parallel to the direction of the slow component the double refraction is negative.

Pleochroism—One other orthoscopic test will be described, namely that for pleochroism. Pleochroism is the property of some substances of absorbing light of different colours in different crystallographic directions. Since ordinary light vibrates in all planes parallel to its direction of propagation, this property requires plane polarized light for its detection or measurement. Copper acetate monohydrate exhibits noticeable pleochroism through the colour range azure blue to bluish green. Acetaldehyde-2:4-dinitrophenylhydrazone exhibits a colour range from pale green to deep yellow. A crystal is tested for pleochroism as follows. The crystal is centred in the field of view and the analyser removed. The polarizer is rotated within its mounting and the crystal watched for a change of colour.

Table II. Remain Dark Through 360°

A. *Give no interference figure in convergent light*	*Isotropic have only one RI*	*(a) Have no regular structure or cleavage*		*Amorphous*
		(b) Show regular form structure or cleavage		*Isometric*
B. *Give optic axis interference figure on convergent light*	*Anisotropic*	*Uniaxial two RI n_o, n_e*	*Tetragonal*	*Positive if $n_o < n_e$*
			Hexagonal	*Negative if $n_o > n_e$*
		Biaxial three RI n_g, n_m, n_p	*Orthorhombic*	*Positive if $n_g - n_m > n_m - n_p$*
			Monoclinic	
			Triclinic	*Negative if $n_g - n_m < n_m - n_p$*

Table III. Remain Light Through 360°

Anisotropic	*Single crystals—normal to optic axis and having high birefringence or dispersion*
	Aggregates—more than one substance present

Table IV. Alternate Light and Dark Through 360°

Anisotropic	*Uniaxial two RI n_o, n_e*	*Tetragonal*	*Positive if $n_o < n_e$*	
		Hexagonal	*Negative if $n_o > n_e$*	
	Biaxial three RI n_g, n_m, n_l	*Common parallel or symmetrical extinction*	*Orthorhombic*	*Positive if $n_g - n_m > n_m - n_l$*
		Both parallel and inclined extinction in plates parallel to axis	*Monoclinic*	*Negative if $n_g - n_m < n_m - n_l$*
		Parallel extinction rare and fortuitous	*Triclinic*	

SUMMARY

It is possible in a brief time to discover the following characteristics of a material by measurement on the polarizing microscope: shape, colour, symmetry, extinction angle, birefringence, pleochroism.

Materials can be allocated to their respective crystal systems from the consideration of their behaviour between crossed polarizers given in *Tables II, III* and *IV*.

When the above information has been obtained almost all crystalline substances may be traced in the Barker Index, or the works of A. N. WINCHELL[8], P. GROTH[9] and others.

THE POLARIZING MICROSCOPE IN BIOLOGICAL RESEARCH

Under the inspiration of F. O. SCHMITT[10], W. J. SCHMIDT[11], H. S. BENNETT[2] and other workers the application of the polarizing microscope to biological studies has been revived. The methods of polarizing light microscopy applied to biology enable information on the fine structure of biological material to be obtained, and enable a quantitative aspect to be introduced into the microscopical biological world. The recent researches[4,5] of A. F. HUGHES, M. M. SWANN and J. M. MITCHISON of the Cambridge School illustrate the results of the revival of interest in the use of polarized light.

GENERAL PRINCIPLES OF POLARIZED LIGHT AS IT AFFECTS BIOLOGICAL INVESTIGATION

Matter is built up of molecules, composed of atoms characterized by one or more electrons disposed about a nucleus; when atoms take up energy the electrons are moved to a higher energy level, and give up this energy as radiant energy when the substance returns to a lower energy level. The vibration of electrons in the molecules of a light-transmitting substance are constrained to certain paths of molecular and atomic structure. Such vibrating electrons interact with oscillating electric fields of parallel disposition such as light; it is this interaction which is exploited by the polarizing microscope to give information about molecular

structure and orientation in a substance. The interaction of light with the electrons in a transmitting substance produces different velocities of transmission of the light—made manifest by changes in refractive index—due to the arrangement of molecules or small particles in the substance or medium. The effect is known as retardation. Sometimes the reaction results in differential absorption of light as the geometry of the light vibration is related to the orientation of structure in the substance. This phenomenon is known as dichroism.

In bodies made up of parallel carbon chains with relatively unimportant side chains, for example collagen, the slow axis of transmission of light is parallel to the long axis of the chain. In objects made up of parallel chains with important side chains, the slow axis of transmission may be transverse to the long axis, as in the nucleic acids. Similarly, in asymmetric structures the polarizability of electrons varies in different directions with consequent differences in the velocity of transmission of light in accordance with the plane of transmission of the electric vector.

Absorption of light in a substance occurs when a beam enters a medium with electrons capable of undergoing transitions to higher energy levels[12] and where the energy change ΔE is related to the frequency of the light ν by $\Delta E = h\nu$ (h being PLANK's constant). The path of the light and vibrating electrons is determined by the electrons and the disposition and shape of the molecules on which the electrons reside[13]. Coloured compounds are therefore materials consisting of molecules having free electrons capable of being excited to a higher energy level by light of visible frequency and in most cases this excitation takes place without affecting the stability of the compound.

ASYMMETRY AND ITS EFFECT ON THE TRANSMISSION OF LIGHT

Light does not travel with equal velocity through all media. The velocity of transmission is dependent on the interaction between the electric vector of the light and the electrons of the molecules of the substance of which the media are composed. If the electronic resonator, which for practical purposes may be considered to be the molecules or the ions of the light-transmitting substance, is distributed at random, then the medium is isotropic. Most glasses, liquids and gases are isotropic because a light ray is constrained on entry by a statistically equal distribution of oscillators (ions or molecules). Most cubic crystals are isotropic because the changes within the crystal are equal and symmetrically distributed.

If the many oscillators or resonators carried by the ions and molecules of a material show preferred directions of symmetry, the medium is anisotropic and will transmit light with different velocities for different directions of propagation. For light of a given wavelength an anisotropic medium has more than one order of refraction and is said to be birefringent, because the molecules and ions are asymmetrically arranged within the medium.

Birefringence is an intrinsic property of a material and is independent of the thickness of the specimen. It is expressed numerically by the difference between the extraordinary and ordinary refractive indices ($\eta_e - \eta_o$). Birefringence is determined by measuring retardation expressed either as the fraction of the wave θ or as a length Γ usually given in Å[10]. The measurement is made by inserting a compensator in the microscopic tube just below the analyser.

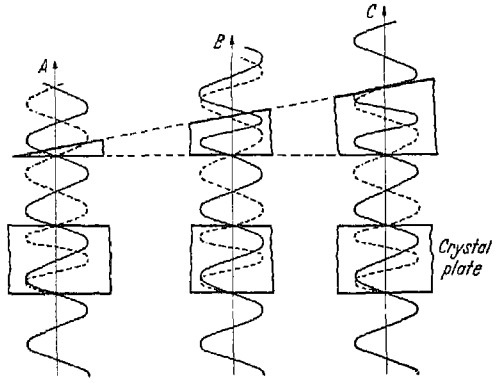

Figure 10. Compensation by quartz wedge (after Wahlstrom[1])

Figure 10 shows how the insertion of a wedge of crystalline quartz can restore the properties of a wave of light which has been altered by passing through an optically reactive medium[1]. Such a wedge can be calibrated to enable measurements of birefringence to be made. The numerical value of birefringence is the phase difference or retardation divided by the thickness of a specimen d

$$\eta_e - \eta_o = \frac{0\lambda}{d} = \frac{\Gamma}{d}$$

In all biological material η_e differs from η_o by very small amounts, so that a birefringence of 0·05 is quite high and birefringences of 0·001 have to be dealt with. Most anisotropic biological material is uniaxial, that is, it possesses one axis which does not show double refraction. This axis may usually be discovered from the general symmetry of the material or object. In fibres the optic axis is usually

the fibre axis. If a cross-section of a muscle fibre section is made and examined in polarized light no birefringence will be detected, demonstrating that the fibre axis is also the optic axis.

The directions in which refractive indices are greatest and least correspond to the characteristic dimensions of the structures. The convention used to describe the birefringence is the same as that used by the chemist and mineralogist, that is if the refractive index parallel to the axis is greater than that for light vibrating perpendicular to it the material is said to have positive birefringence. For example protein fibres and carbohydrate fibres are positive uniaxial and fibres of nucleoproteins and fibres containing nucleic acid in lipids, for example phosphatides, cerebrosides and soaps, are negative uniaxial. The optic axis is parallel to the chains and perpendicular to the plane of a layer.

In compounds sign gives the relative amount of each material; in a lipid protein the relative amount of lipid may be determined by the increase in positive birefringence, with respect to the fibre axis, after soaking in a lipid solvent.

The above methods are of great use in the study of microscopic objects such as intracellular organelles.

There are several methods of measuring retardation; large retardations, which are of greatest interest to the chemist and mineralogist, are best measured directly with a gypsum plate. The best method of measuring small retardations, which are of greatest interest to the biologist, is with a null method such as that obtained by the use of a Kohler compensator, one version of which is made in Great Britain by Cooke, Troughton and Simms.

TYPES OF BIREFRINGENCE

There are three types of birefringence. Biological material may exhibit one, or combinations of two or of all three types in any single specimen.

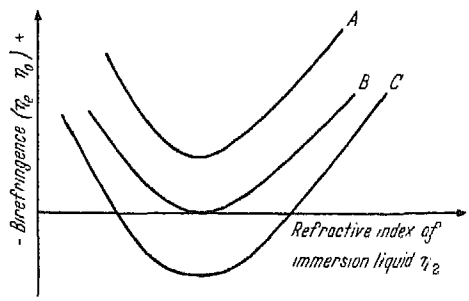

Figure 11 (after Schmitt[10])

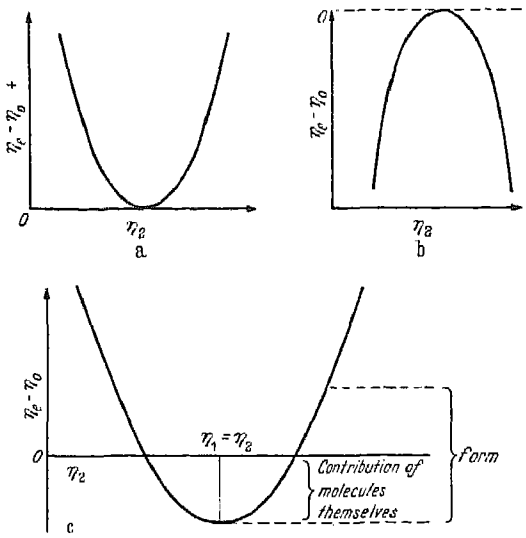

Figure 12. Form of material: a *rods;* b *disks;* c *flakes (after Bennett[2])*

Crystalline birefringence—This is due to a regular anisotropic alignment of chemical bonds, ions or molecules as in crystalline material. It is independent of the surrounding medium, the structure being so closely packed that liquid cannot penetrate between the molecules, for example collagen fibres, chromosomes, muscle fibres, and cellular fibres.

Form birefringence—This is due to the asymmetrical shape of the ultimate particles of which the specimen is composed. If the particles are small with respect to the wavelength of light used and are preferentially orientated the system will usually show form birefringence. The birefringence disappears when the refractive index of the particles is equal to that of the surrounding medium.

Determination of the sign of form birefringence will indicate the direction in which the particles are orientated, *i.e.* will indicate whether the particles are rods or flakes. It is usual to construct a graph in which birefringence is plotted against the refractive index of the immersion liquid, using a series of immersion liquids of known refractive index. *Figure 11* shows the type of results obtained[10]. In curve *A* the molecules show positive crystalline birefringence. Curve *B*, in which the minimum of the curve occurs at zero birefringence, represents a material composed of particles with molecules that are not regularly arranged. Curve *C* shows a substance the constituent molecules of which show negative crystalline birefringence. All these materials show a positive general form.

H. S. BENNETT gives[2] an even more illuminating series of $(\eta_e - \eta_o)$ to η_2 curves (see *Figure 12*) in which the form of the curve provides information on the type of material, rod, flake *etc*, under examination.

Strain birefringence—This is produced when systems are subjected to mechanical forces. If the system contains imperfectly orientated particles the orientation and therefore the birefringence will be increased by tension or compression. The maximum birefringence is reached when the maximum possible orientation of the particles is reached. The stretching of gelatine or celluloid is a well known illustration of strain birefringence. Biological material showing strain birefringence is to be found in stretched connective tissue. There are other forms of birefringence formed by combinations of the above and by optical rotation. The polarizing microscope also yields information on symmetry to the biologist as it does to the mineralogist.

CONCLUSION

Because of the different nature of the information required by the two groups of workers some repetition has been necessary. The chemist and mineralogist require measurements which will enable a morphology of the substance under examination to be constructed; this leads to its identification from indexes such as the Barker Index of crystals. The approach is best covered by the older geometrical and physical optics. The biologist on the other hand is interested in the ultimate fine structure of his material, information which it is difficult to obtain by x-ray diffraction owing to the large amount of hydrogen present in biological substances and the great complexity of their architecture. Electron microscopy is limited at present to desiccated material. Although the concepts of L. PAULING[14] and other physical chemists on the constitution of matter and its interaction with electromagnetic waves have become of greater use than the older geometrical optics, the polarizing microscope offers a valuable contribution to methods of investigation.

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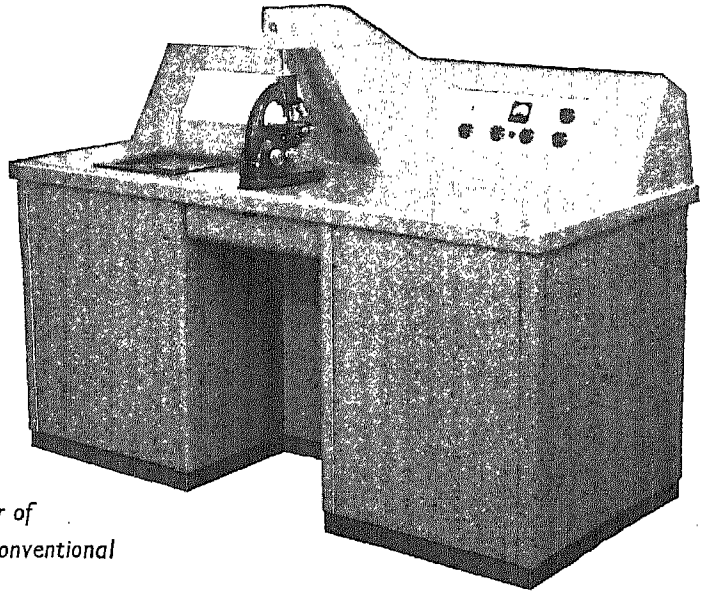
FLYING-SPOT PARTICLE RESOLVER



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